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Preface

The UJNR (The U.S.-Japan Cooperative Program in Natural Resources) Aquaculture Panel was established in 1968, and the business meeting and symposium have been held every year since 1971. Through the long history of UJNR, Aquaculture Panel has contributed to the development of aquaculture researches of both countries by means of various cooperative activities, such as the exchange of scientists and literatures, and the promotion of joint research projects. The Aquaculture Panel is highly appraised as one of the most active UJNR panels in both countries.

The 47th business meeting of the UJNR Aquaculture Panel was conducted at Okinawa Industry Support Center, Okinawa prefecture, Japan on November 12, 2019, and the scientific symposium was held at the same venue from November 12 to 13. The symposium theme was "Marine aquaculture in a changing environment" which was the 10th three-year plan commenced in 2017. As the final year of the three-year plan, we discussed about application of aquaculture technology to provide sustainable seafood and reduce impacts of environmental change. Seventeen oral presentations were made on topics such as new techniques to assess and mitigate impacts of environmental changes on aquaculture, impact assessment of environmental changes and conservation of fisheries environment in coral reef areas, production management under environmental changes in bivalve aquaculture, and aquaculture technologies to respond to environmental changes in seaweed breeding and feed development. The proceedings of the 47th UJNR Aquaculture Panel scientific symposium "Marine aquaculture in a changing environment" is published as the special issue of the Bulletin of Japan Fisheries Research and Education Agency. With great pleasure, this UJNR proceedings containing high quality papers authored by selected American and Japanese aquaculture scientists will hopefully help improve the aquaculture environment programs, which is expected to contribute to the development of the aquaculture industry in both the United States and Japan.

Finally, I would like to express my sincere gratitude to the colleagues involved in the UJNR Aquaculture Panel for their efforts in the preparation and organization of the symposium. I would also like to deeply thank the editorial board members for publishing the proceedings.

Hideaki Aono, Ph.D.
Chair of UJNR Aquaculture Panel
Executive Director
Japan Fisheries Research and Education Agency

Participants in 47th UJNR Aquaculture Panel Symposium, held in Okinawa Industry Support Center, Okinawa, Japan, November 12–13, 2019

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Program

The 47th Scientific Symposium of UJNR Aquaculture Panel

Marine Aquaculture in a Changing Environment

Date:

November 12　9:15 - 12:00
November 13　9:15 - 17:00

Venue:

Okinawa Industry Support Center, 1831 - 1 Oroku, Naha, Okinawa

Aim of the Symposium

The UJNR Aquaculture Panel is a cooperative research exchange between the U.S. and Japan jointly addressing environmental and technical issues that affect the aquaculture industries of both nations.

The 47th UJNR Aquaculture Symposium is the final symposium of a 3-year cycle with the theme Marine Aquaculture in a Changing Environment. Environmental changes impact aquaculture in many ways. Nutrient pollution is driving eutrophication and dead zones; ocean acidification is changing water chemistry, and climate change is already influencing our food supply, freshwater availability, weather and way of life. Aquaculture will be impacted by, and can also impact, these environmental changes over various scales. Aquaculture of finfish, shellfish and seaweed have different threats, benefits and opportunities related to environmental change.

Over the last two years, we discussed the potential of aquaculture to mitigate impacts of environmental change (*e.g.* carbon sequestration, bioextraction of nutrients and CO_2, antacidity, and oxygen production), impacts of environmental change on aquaculture production (*e.g.* effects of ocean acidification on shellfish aquaculture), and science to mitigate these impacts (countermeasures). This is the final year of the three year-plan, and the symposium theme is the Application of Aquaculture Technology to Provide Sustainable Seafood and Reduce Impacts of Environmental Change. This theme includes development of technology to increase marine aquaculture production to offset seafood deficit due to loss of capture fisheries impacted by environmental change, and to augment food deficits due to impacts of environmental change on inland agriculture.

Tuesday, November 12, 2019

Registration: 9:00 - 9:15

Opening Session

Welcome and Aim of the Symposium

Hideaki Aono (Japan Panel Chair, Japan Fisheries Research and Education Agency)····· 9:15 - 9:25

Session I. New techniques to assess and mitigate impacts of environmental changes on aquaculture

(Moderators: Simona Augyte and Natsuki Hasegawa)

1. Bacterial communities in marine sediments; a biological parameter to evaluate coastal environment
   Tomoko Sakami (National Research Institute of Aquaculture, FRA) ····· 9:25 - 9:55

2. Applications of environmental DNA data in support of aquaculture
   Tom Noji (Northeast Fisheries Science Center, NOAA Fisheries) ····· 9:55 - 10:25

3. Stable isotopic approach to investigate nitrogen pathway in coastal aquaculture area
   Satoshi Watanabe (National Research Institute of Aquaculture, FRA) ····· 10:25 - 10:55

4. Establishment of immunological assays and baseline profile of hemocytes in the hard clam *Mercenaria mercenaria* as evaluation biomarkers for environmental stresses
   Huiping Yang (School of Forest Resources and Conservation, Institute of Food and Agricultural Sciences, University of Florida) ····· 10:55 - 11:25

5. Purple urchin barrens: an opportunity for aquaculture and fisheries to work together to solve an environmental issue
   Luke Gardner (California Sea Grant) ····· 11:25 - 11:55

Wednesday, November 13, 2019

Session II. Impact assessment of environmental changes and conservation of fisheries environment in coral reef areas

(Moderators: Luke Gardner and Masakazu Hori)

6. Impacts of ocean acidification on Japan coastal water and marine fisheries
   Haruko Kurihara (Invited speaker)(University of the Ryukyus) ····· 9:15 - 9:45

7. Sustain seafood resources in the U.S. affiliated Pacific islands- status and strategies
   Cheng-Sheng Lee (Center for Tropical and Subtropical Aquaculture, United States Department of Agriculture) ····· 9:45 - 10:15

8. Towards effective coral community restoration for sustainable fishery of a coral reef grouper *Epinepherus ongus*: implication of ecosystem-based management
   Atsushi Nanami (Seikai National Fisheries Research Institute, FRA) ····· 10:15 - 10:45

9. Scaling up coral restoration to meet the demands of a collapsing ecosystem
   Tali Vardi (ECS for NOAA Fisheries Office of Science & Technology) ····· 10:45 - 11:15

10. Sustainable large-scale coral restoration by establishing "artificial spawning hotspot"
    Go Suzuki (Seikai National Fisheries Research Institute, FRA)····· 11:15 - 11:45

Lunch Break ····· 11:45 - 13:00

Session III. Production management under environmental changes in bivalve aquaculture

(Moderators: Cheng-Sheng Lee and Atsushi Nanami)

11. The influence of climate and environment on the growth and survival of Pacific oyster seed in US West Coast estuaries
    Brett Dumbauld (Agricultural Research Service, United States Department of Agriculture) ····· 13:00 - 13:30

12. Comparative study of the impact of environmental changes on oyster culture between USA and Japan, as collaborative research under UJNR
    Natsuki Hasegawa (Hokkaido National Fisheries Research Institute, FRA) ····· 13:30 - 14:00

13. Oyster aquaculture using seagrass beds as a climate change countermeasure
    Masakazu Hori (National Research Institute of Fisheries and Environment of Inland Sea, FRA) ····· 14:00 - 14:30

Coffee Break ····· 14:30 - 14:45

Session IV. Aquaculture technologies to respond to environmental changes; Seaweed breeding and feed development

(Moderators: Brett Dumbauld and Satoshi Watanabe)

14. Kelp, *Saccharina* spp, population genetics in the Northwest Atlantic for guiding a breeding program of thermally resilient strains

    Simona Augyte (Dept. of Ecology and Evolutionary Biology, University of Connecticut) 14:45 - 15:15

15. Cell selection technique for establishment of low salinity tolerance strain in *Pyropia tenuipedalis*

    Mahiko Abe (Applied Aquabiology, National Fisheries University, FRA) ····· 15:15 - 15:45

16. Improvement of dietary effect on juvenile *Ruditapes philippinarum* using the dietary-supplements and new diet microalga

    Yasuhiro Yamasaki (Applied Aquabiology, National Fisheries University, FRA) ····· 15:45 - 16:15

17. Exploration of alternative protein sources in the development of a sustainable Japanese white trevally *Pseudocaranx dentex* juvenile diet

    Jonas Miller (Uragami Station, Aquaculture Research Institute, Kindai University) 16:15 - 16:45

Science Symposium Closing

Michael Rust (US Panel Chair, NOAA Fisheries Office of Aquaculture) ····· 16:45 - 17:00

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Bacterial community composition of the sediment in the coastal regions of Kashima-Nada and Kujukurihama, Japan

Tomoko SAKAMI[*1] and Toru UDAGAWA[*2]

Abstract: Environmental monitoring of marine coastal areas is becoming increasingly important because of accelerated global climate change and rapid industrial development. The composition of the bacterial community in bottom sediments is strongly related to the surrounding environment. Thus, surface sediments were collected at the coastal regions of Kashima-Nada and Kujukurihama to elucidate how bacterial communities vary where the confluence of the cold Oyashio and warm Kuroshio Currents occur. The bacterial communities collected at water depths of 10 m were different between the Kashima-Nada and Kujukurihama areas, but were not different at water depths of 30 m. The chlorophyll *a* content in the sediment and bottom water temperature were indicated as the major environmental parameters influencing the composition of the bacterial communities. At 10 m depth in the Kujukurihama area, operational taxonomic units (OTUs) of *Bacteroides*, which are known as high molecular weight organic matter degraders, were abundant, whereas OTUs of Nitrospinae and *Nitrosospira*, which are related to the nitrification process, were less abundant. The taxonomic groups characteristic of the sediment seemed to be related to the properties of organic matter quality in the sediment.

Key words: bacterial community composition, sediment quality, Kashima-Nada, Kujukurihama

Introduction

Terrestrial materials such as soil, inorganic nutrients, and organic matter are discharged into the sea through river water flow or by land erosion, with considerable consequences for the coastal environment (Bergamaschi *et al.*, 2012). In addition to industrial development on land, global climate change, which is predicted to bring about increases in torrential rain and storms (Yagi *et al.*, 2015), will change material inputs from land to coastal areas (Philippart *et al.*, 2011; Viitasalo *et al.*, 2015). To deal with prospective environmental changes, we need to monitor certain environmental parameters precisely. Using physical and chemical monitoring techniques, it is now possible to obtain highly accurate data owing to advances in measuring equipment and information technology (Bean *et al.*, 2017). However, monitoring physical and chemical parameters is not enough to evaluate integrated environmental conditions for living organisms. As a biological parameter, macrobenthos fauna are often examined in order to monitor coastal environmental conditions. This requires time and labor, and the amount of data is less than what is obtained by physical and chemical monitoring. In contrast, approximately one hundred million bacterial cells can be observed in a single gram of marine sediment (Schmidt *et al.*, 1998). Data collection for bacterial community composition analysis is a mechanical process that augments physical and chemical parameter measurement. Typically, millions of bacterial sequences can be retrieved from one sample, and they can be clustered into hundreds or thousands of species. Therefore, it may be possible to detect certain integrated environmental changes which

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cannot be detected by physical and chemical parameters alone.

Bacterial community composition of coastal bottom sediments is more diverse than that of seawater (Zinger *et al.*, 2011). Community compositions are often related to environmental conditions such as the particle size of the sediment, and the dissolved oxygen concentration in the bottom water (Zheng *et al.*, 2014), or the organic matter content in the sediment (Qiao *et al.*, 2018). Salinity change in the brackish water area (Vidal-Durà *et al.*, 2018) and anthropogenic contamination, such as metal and polycyclic aromatic hydrocarbon contaminants from land (Sun *et al.*, 2013), can also affect sediment bacterial communities. In land soil, bacterial communities are more affected by the biochemical conditions of the soil, such as organic matter content, than by the origin or geographic location of the soil (Lin *et al.*, 2019). In marine bottom sediments, however, it is not clear what difference the sea area makes. Factors such as the geographical distance from land, or overlying seawater characteristics (the current type), may affect bacterial communities together with the bottom environmental parameters such as particle size or organic matter content.

Kashima-Nada and Kujukurihama are open, large, sandy beaches, which are located at the northern and southern parts of Cape Inubo, respectively, on the Pacific coast of Japan. In these areas, primary production is high, and there are high fishery catches of bivalves and whitebait (Okunishi *et al.*, 2000). The cold Oyashio Current and the warm Kuroshio Current run along the Pacific coast of Japan, and they meet at a confluence point, off Cape Inubo. It is well known that the biological community of the Pacific coast of Japan changes, with Cape Inubo marking the border of the northern and southern fauna and flora (Shimizu, 2001). In addition, the Tone river mouth, which is the largest river in Japan, is north of Cape Inubo. Together with the large amount of river water discharge and the high wind in this area, a strong tidal stream occurs around Cape Inubo, and complex water exchange and sediment transportation occurs (Yagi *et al.*, 2002; Arai *et al.*, 2006; Uzaki *et al.*, 2016). The distribution patterns of macrobenthos fauna vary within a certain band north and south of Cape Inubo, and the exact dimensions of this band depend also on the year of the survey (Shimizu, 2001). Bacterial communities in sediments may change in different ways from the larger organisms and may help elucidate bottom environmental conditions in more detail.

We examined bacterial community composition in the sediments collected at Kashima-Nada and Kujukurihama by using 16S ribosomal RNA (rRNA) gene sequences. By analyzing relationships between the community composition and sediment quality parameters, such as organic matter content and mud content, we aimed to clarify how the bacterial community varies in this area, and how the sediment qualities influence or reflect these variations.

Materials and Methods

Sample collection

Sediment samples were collected at three sites adjacent to the shorelines at a water depth of 10 m and 30 m, at the Kashima-Nada and Kujukurihama beaches in July, August, and September 2017 (**Fig. 1**). The annual water temperatures ranged from 25℃ (highest; range: 23–27℃) to 12.5℃ (lowest; range: 9–14℃) at Kashima-Nada, and from 26℃ (highest; range: 25–27℃) to 15℃ (lowest; range: 12–16℃) at Kujukurihama area between 2014 and 2018 (Tokyo District Meteorological Observatory). A Smith-

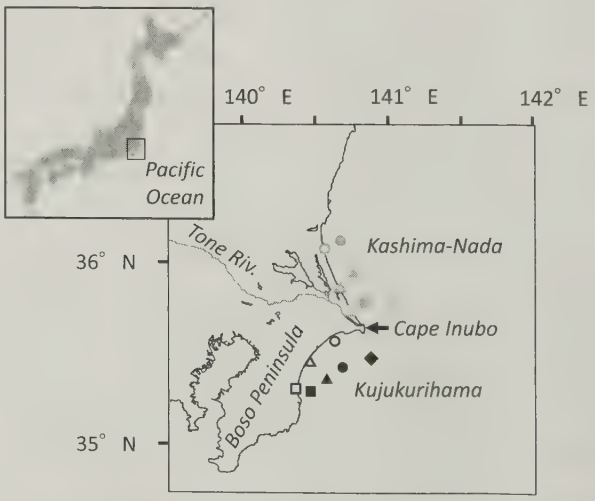

Fig. 1. Sample collection points at the coastal regions of Kashima-Nada and Kujukurihama on the Pacific coast, Japan. Open symbols indicate 10 m depth, and solid symbols indicate 30 m depth.

McIntyre grab sediment sampler (1/20 m^2, RIGO, Osaka, Japan) was used to collect sediment samples. A surface sediment sample (top 2 cm) was collected using a plastic corer of 23 mm diameter, and the sample was frozen immediately in a sterilized plastic bag. The sediment samples were kept at −60℃ until further processing. Temperature and salinity were measured using a multi-parameter handy conductivity temperature depth profiler (RINKO Profiler, ASTD152, JFE Advantech, Nishinomiya, Japan).

Sediment quality analysis

The following variables were analyzed: (i) particle size was determined using a laser diffraction particle size analyzer (SALD-3100; Shimadzu, Kyoto, Japan), (ii) ignition loss was evaluated by igniting the sediment samples at 550℃ for 12 h, (iii) chlorophyll *a* content was determined by fluorescence using a spectrophotometer (10AU, Turner Designs, San Jose, USA).

DNA extraction and sequencing

DNA was extracted from a 0.4 to 0.6 g sediment sample using the Fast DNA SPIN Kit for soil (MP Biomedicals LLC., Ohio, USA) according to the manufacturer's instructions. Barcodes were attached to DNA fragments in a 2-step PCR amplification that targeted the variable V1/V2 region of the 16S rRNA gene based on the protocol of Sakami *et al.* (2016). First PCR was conducted using 27Fm and 338R primers (Kim *et al.*, 2013), then the sequences were modified by adding 34 and 36 bp snippets, respectively, in order to later add molecular identifier (MID) tags. Pooled amplicons were paired-end sequenced (2×300 bp) on the Miseq device (Illumina, Tokyo, Japan).

Bacterial community analysis

The sequences obtained by extraction were quality filtered, and chimeras were removed using the UCHIME algorithm (Edger *et al.*, 2011) with alignment to the SILVA database (SSU_Ref_NR ver. 119) reference alignment. Sequences that passed through the filtering process were divided into operational taxonomic units (OTUs) using a 3% similarity threshold and were identified with taxonomic annotation using the Mothur Miseq standard operating procedure (SOP) (Kozich *et al.*, 2013). Inverse Simpson diversity index was also estimated using this procedure.

The analyses below were done using the statistical package R version 3.5.3 (R Core Team, 2018). Detrended correspondence analysis was performed and produced an axis length of less than four, indicating a linear relationship between community composition and the examined environmental parameters: temperature, salinity, median particle diameter, mud content, ignition loss, and chlorophyll *a* content. Therefore, relationships of the bacterial community composition across the sampling sites were examined using non-metric multi-dimensional scaling (NMDS) analysis, and relationships of the bacterial community composition and environmental parameters were examined using redundancy analysis (RDA). Relative abundance of subphyla of the top three phyla, namely Proteobacteria, Bacteroidetes, and Firmicutes, and phyla whose relative abundance was more than 0.001 were compared among four sampling areas: Kashima-Nada-10 m, Kashima-Nada-30 m, Kujukurihama-10 m and Kujukurihama-30 m. A heatmap figure was made using the 16 taxonomic groups that were significantly different among the four areas (ANOVA, $p < 0.05$).

Results

Environmental parameters

Median particle diameter (d50) was larger in the Kashima-Nada-30 m, and mud content was higher in Kujukurihama-30 m. Ignition loss was higher at Kujukurihama at a depth of 10 m and lower at Kashima-Nada at a depth of 30 m. Chlorophyll *a* content was higher at a depth of 30 m in both areas.

Bacterial community composition

The number of sequences obtained from sediment profiles ranged from 60,000 to 138,000 per sample. The community coverage was estimated to be more than 0.999 for all samples. The sequences were clustered into 803 operational taxonomic units (OTUs). Community diversity (Inverse Simpson index) ranged from 5.8 to 11.4, and it tended to be

Table 1. Average values of environmental parameters and bacterial community diversity at the sampling areas

	Temp (°C)	Salinity (psu)	Central particle diameter (um)	Mud Content (%)	Ignition Loss (%)	Chlorophyl *a* content (ug/cm^2)	Invsimpson index
Kashima-Nada -10m	20.7^{ab}	33.7^{b}	187^{b}	0.18^{b}	1.4^{bc}	56.3^{b}	6.8^{b}
Kashima-Nada -30m	17.0^{b}	33.8^{b}	239^{a}	0.05^{b}	1.4^{c}	203^{a}	7.4^{ab}
Kujukurihama -10m	21.6^{a}	34.0^{ab}	166^{b}	0.36^{b}	2.1^{a}	88.8^{b}	8.4^{ab}
Kujukurihama--30m	19.3^{ab}	34.3^{a}	160^{b}	0.99^{a}	1.8^{ab}	167^{a}	8.9^{a}

* Numbers with different superscripts are different at the significance level of $p < 0.05$.

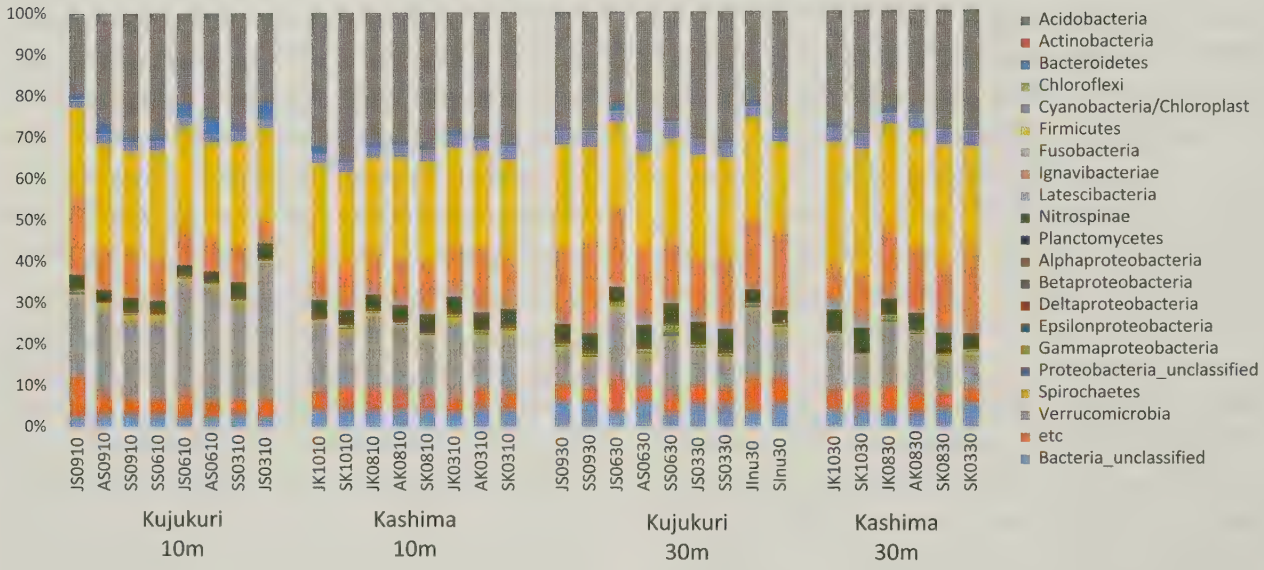

Fig. 2. Bacterial community composition in the surface sediment collected at 10 and 30 m depths off the coastal regions of Kashima-Nada and Kujukurihama in July, August and September, 2017.

high at Kujukurihama (**Table 1**). Of the OTUs, 33% to 53% belonged to the phylum, Proteobacteria, and the major subphyla were Gammaproteobacteria (21%–30%) and Deltaproteobacteria (5%–21%). Other major phyla were Bacteroidetes (7%–33%), Actinobacteria (3%–9%) and Acidobacteria (2%–6%) (**Fig. 2**).

The community composition of each sample was plotted in two dimensions following NMDS (non-metric multi-dimensional scaling) analysis (**Fig. 3**). Bacterial communities at a depth of 30 m at both Kashima-Nada and Kujukurihama were plotted together in the top area of the graph. Bacterial communities at a depth of 10 m at Kashima-Nada were plotted below the 30 m communities, and those off Kujukurihama were plotted in the bottom area of the graph.

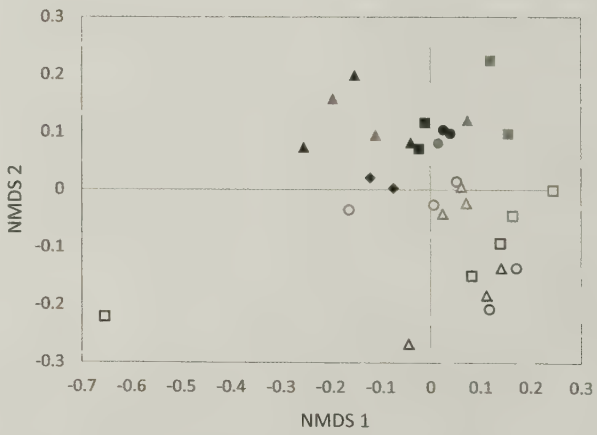

Fig. 3. Non-metric multi-dimensional scaling plot of sediment bacterial communities with environmental parameters. Black symbols indicate Kujukurihama area, and gray symbols indicate Kashima-Nada area. Open symbols indicate a depth of 10 m and solid symbols indicate 30 m depth.

The relationships between the composition of the bacterial community and environmental parameters were examined using redundancy analysis (RDA) (**Fig. 4**). The X-axis represented 54% of the community variation. Bacterial communities were placed in order of the areas of 30 m depth, the 10 m depth at Kashima-Nada, and the 10 m depth at Kujukurihama from the right. The Y-axis represented 4% of the community variation. Bacterial communities at a depth of 10 m at Kujukurihama were placed on this axis. Large directional arrows of chlorophyll *a* content and water temperature were along the X-axis, implying that these parameters were strongly related to variation in bacterial community composition. Small directional arrows for mud content, salinity, and ignition loss were on the lower right. No obvious directional arrows were observed along the Y-axis.

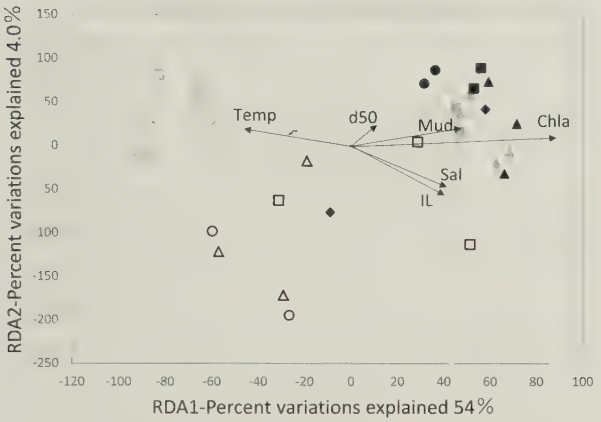

Fig. 4. Redundancy analysis (RDA) of sediment bacterial communities with environmental parameters. Black symbols indicate Kujukurihama area, and gray symbols indicate Kashima-Nada area. Open symbols indicate 10 m depth, and solid symbols indicate 30 m depth. IL, Chl a, d50, and mud represent ignition loss, chlorophyll *a* content, median particle size, and mud content, respectively.

The relative abundance of the major taxonomic groups, whose abundance was different among the four sea areas, was shown in a heat map (**Fig. 5**). The taxonomic groups were clustered into two groups (cluster I and II), with the first cluster divided further into three subgroups (cluster Ia, Ib, Ic). Cluster Ia included Acidobacteria and Proteobacteria, except for *beta* and *epsilon* types, and abundance tended to be low at a depth of 10 m off the Kujukurihama coast. Cluster Ib included Chloroflexi and Ignavibacteriae, and the abundances tended to be low at a depth of 30 m at Kashima-Nada and at Kujukurihama. Cluster Ic included Betaproteobacteria, Planctomycetes,

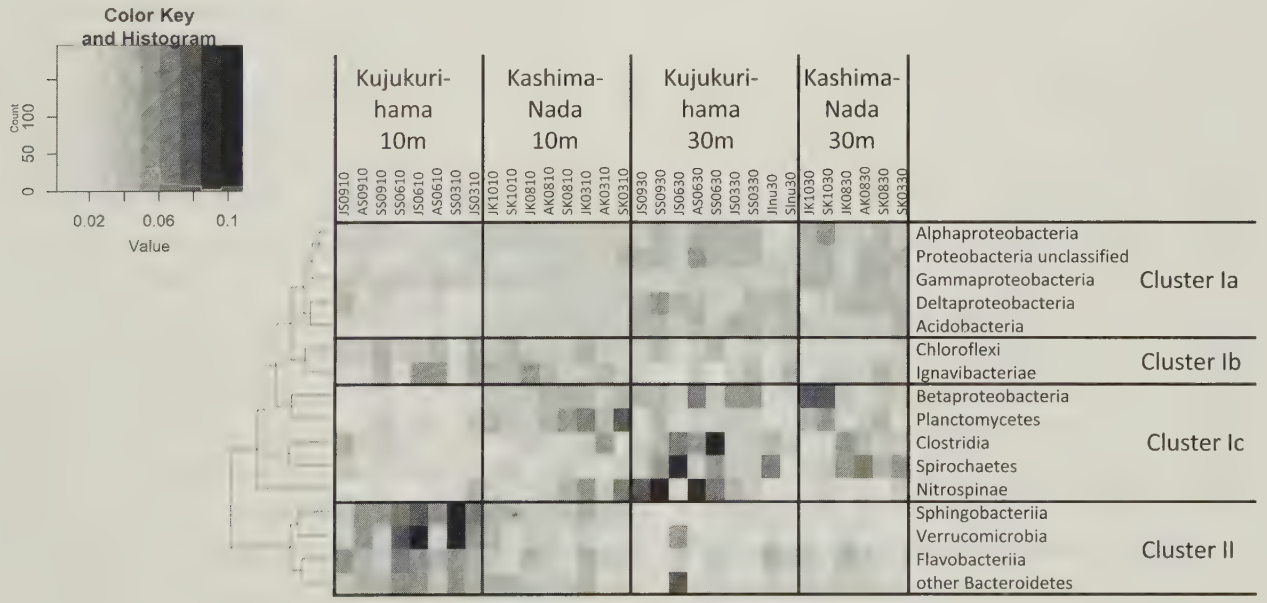

Fig. 5. Hierarchically clustered heatmap of bacterial communities from the four areas at the phylum or subphylum level. Rows represent the relative abundance of each bacterial group, and columns indicate different sediment samples. The relative abundance of each bacterial taxon is depicted by color intensity with the legend indicated at the top of the figure.

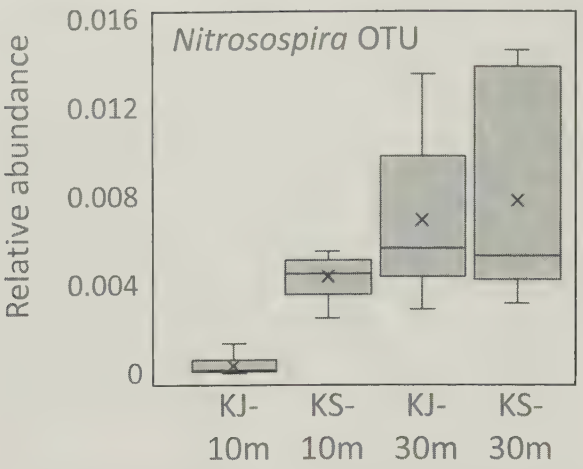

Fig. 6. Relative abundance of a *Nitrosospira* OTU in the four areas. KJ indicates Kujukurihama and KS indicates Kashima-Nada.

Clostridia, Spirochetes, and Nitrospinae, and the abundance tended to be low at 10 m depths in the Kujukurihama area. Relative abundance of an OTU affiliated to genus *Nitrosospira* accounted for 73% of betaproteobacterial OTUs. It was low at a depth of 10 m at Kujukurihama (**Fig. 6**). Cluster II included Flavobacteriia, Sphingobacteriia, other Bacteroides, and Verrucomicrobia, and the abundance tended to be high at 10 m depth in the Kujukurihama area.

Discussion

It is well known that the fauna and flora change at the border of the northern and southern junction at Cape Inubo, on the Pacific coast of Japan. For example, the distribution of marine bivalves and snails changes from tropical to cold-water varieties at the border of the Boso Peninsula (Shimizu, 2001). The warm Kuroshio Current which runs along the western Japanese Pacific coast changes direction to the east at Cape Inubo, leaving the main island of Japan. Therefore, the Kujukurihama area is influenced by the warm Kuroshio Current. In contrast, a confluence of the warm Kuroshio and the cold Oyashio Currents occurs at the northern end of Cape Inubo, the Kashima-Nada area. Moreover, the four examined areas, *viz.* the 10 m and 30 m depths at the Kashima-Nada and Kujukurihama sites, had different bottom environments as indicated by their particle size and organic matter content data. The bacterial community composition was different between Kashima-Nada and Kujukurihama at depths of 10 m, but no obvious difference was observed at 30 m. The water depth, chlorophyll *a* content, and mud content was related to the variation in bacterial community composition in addition to the water temperature, indicating that the bacterial communities present in the examined areas varied primarily with the conditions of the bottom environment rather than with the differences in current. On land soil, bacterial community structure and diversity were shown to be affected more by parent material differences or biogeographical separation of the soil than the soil properties, such as organic matter content (Lin *et al.*, 2019). In marine ecosystems, there is evidence that the benthic bacterial community composition is affected more by the geographic location than the ecosystem type and time or upper water productivity (Zinger *et al.*, 2011). Since the bacterial community compositions were affected by sediment qualities rather than the geographical distance, we suggest that there are considerable exchanges of sediment materials between the northern and the southern areas of Cape Inubo.

It has been shown that bacterial community composition in marine bottom sediments changes according to the primary production in the sea (Zinger *et al.*, 2011). The Tone River water discharge to the north side of Cape Inubo, increases primary productivity in both the Kashima-Nada and Kujukurihama areas, and the influence fluctuates widely with precipitation and wind conditions (Arai *et al.*, 2006). The primary productivity in the Kashima-Nada area was estimated to be 0.4 g C/m^2/day, and 76% of the production was estimated to depend on the regeneration of the nutrient due to organic matter degradation within the area (Okunishi *et al.*, 2000). Although primary production and a contribution from regeneration production were not indicated at the Kujukurihama area, the taxonomic features observed in the bacterial community composition may indicate different biological production processes between the two areas.

Concerning the abundance of bacterial taxonomic groups in the four sea areas, cluster II (which was abundant at 10 m at Kujukurihama)

included Flavobacteriia, Sphingobacteriia and Verrucomicrobia, which belong to Bacteroidetes. Bacteroidetes are generally considered to be specialists in the degradation of high molecular weight organic matter (Thomas *et al.*, 2011). The total organic matter (ignition loss) was high, and the chlorophyll *a* content was low at the depth of 10 m at Kujukurihama, suggesting that the sedimentary organic matter was relatively poor in fresh material. Flavobacteriia are often observed abundantly at the end of phytoplankton blooms (Teeling *et al.*, 2012; Sakami *et al.*, 2016). They may degrade not only fresh phytoplankton materials, but also labile, high molecular weight organic matter, which is deposited in the sediment. Among Bacteroidetes, Cytophagaceae members often digest macromolecules such as polysaccharides and proteins (McBride *et al.*, 2014), but their abundance did not differ among the four areas with different availability of these nutrients. Verrucomicrobia are also reported to be candidates for the degradation of polysaccharides in Arctic seawater (Cardman *et al.*, 2014). The cluster Ic was less abundant in the shallow area off Kujukurihama, and Betaproteobacteria and Nitrospinae were included in the cluster. More than 70% of the Betaproteobacterial OTUs belonged to *Nitrosospira* which are known as ammonia oxidizers. Because Nitrospinae is also related to the nitrification process, the nitrification process in this area might be less active than in the other three areas.

In conclusion, the composition of bacterial communities in coastal sediments varied mainly with water depth around Cape Inubo, and the differences were concomitant with differences in some environmental parameters. Moreover, the taxonomic groups characteristically found in an area suggest that the difference seems to be related to the properties of the organic matter of the sediment. Information on the benthic bacterial community is useful for monitoring coastal environmental changes because not only does it reflect the environmental conditions, but its taxonomic features indicate the ecological functions of the community. Future longer-term observations may provide detailed information on coastal environment changes in the area.

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The distributions of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) were determined along an environmental gradient from the coastal mud to the offshore coarse sand at Sendai Bay, Japan. The abundance of AOA ammonia monooxygenase alpha subunit gene (*amoA*) was high in the coastal muddy areas and low in the offshore sandy areas. There was a strong positive correlation between AOA-*amoA* abundance and ammonia content in the sediment. The distribution of AOB-*amoA* was similar to that of AOA-*amoA* in July, but remarkably low in the muddy sediments in December. Clone library analysis indicated that the community composition for both types of organisms differed in sandy and muddy sediments and that the diversity was considerably lower in the muddy sediments. These results suggest that the abundance of ammonia-oxidizing organisms was controlled by the ammonia levels in the sediment. However, there are some inhibitive conditions for AOB: presumably, the low organic matter supply to the surface oxic layer during autumn in the muddy sediment in Sendai Bay.

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Applications of environmental DNA data in support of aquaculture

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Abstract: With advances in analytical and computational technologies the data for environmental DNA or eDNA are becoming rapidly and increasingly available. eDNA data have been applied successfully to assess presence of fish species, impacts of human activities on benthic biota and more recently to a limited extent to assess biomass. Because of the relative ease with which eDNA data can be collected, the number of proposed applications is increasing rapidly; this includes applications to support aquaculture. Some of the applications of particular interest for aquaculture operations include measurement of eDNA as a surveillance tool for pathogens, protected species and escapees from *e.g.* net pens; as an indicator of benthic impacts and efficacy of stock enhancement operations; and health of cultured species.

Key words: environmental DNA, genetics, aquaculture, seafood, production

Introduction

Environmental DNA or eDNA is collected from the environment and not directly from organisms larger than about half a micrometer in size, as determined by pore size of filters used. The collection, extraction and analysis of eDNA have become a popular method in recent years. This was largely made possible by the development of technologies to analyze the genetic sequences of DNA and to the high-powered computing systems easing the analysis of the large data sets acquired.

Aquaculture in Japan is a well-established and thriving industry with an annual production in 2017 of about 700 thousand tons of freshwater and marine animal products; most of this is seafood. In Japan another approximately 500 thousand tons of aquatic plants are also produced annually (http://www.fao.org/fishery/countrysector/naso_japan/en). In contrast in the USA freshwater and marine aquaculture produced only 270 and 41 thousand tons, respectively, in 2017 and ranked 17th in global aquaculture production. Ironically the USA is the leading global importer of fish and fishery products; 90% of USA seafood is imported and half of this is from aquaculture.

Not surprisingly there is now a strong expectation that marine aquaculture will be a significant factor in increasing the USA's seafood productivity, thereby reducing the USA's reliance upon import of seafood and helping to assure food security for the nation. Toward this end, multiple applications exist for eDNA data to support aquaculture to increase seafood production in an environmentally friendly and sustainable manner. This short paper is a summary of a presentation at the US-Japan Bilateral Meeting in Okinawa, Japan, in 2019 and gives a brief overview of some of those applications.

Why environmental DNA?

There are several benefits, which make eDNA a promising research tool. Firstly, the sampling is non-invasive or only minimally invasive. If you can collect water, you can collect eDNA, *e.g.* from rivers, lakes, bays, surface and deep-sea water, and pore

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water in sediments. Further the set of eDNA data comprises a comprehensive overview of all living organisms which contributed DNA into the sampled volume of water in the recent past (generally up to a few days). Moreover, the methodologies for filtering the water, extracting the DNA, amplifying it, and sequencing the genetic material have advanced to the point that eDNA measurements are routine and taught even in high school courses. That said, there is continual (a) technological development to improve the sequencing and amplification of genetic material, (b) increasingly high powered computing systems to analyze millions of data points into Operational Taxonomic Units or OTUs, and (c) expanding reference libraries to correlate OTUs with species.

Quantitative polymerase chain reaction or qPCR can perform species-specific analyses on eDNA to provide quantitative findings of the target species. In contrast, next generation barcoding or metabarcoding provides an estimate of the relative abundance of the suite of organismal DNA collected on your filter. One of the major questions for researchers today is whether metabarcoding of eDNA can be used for quantitative species assessments. Another question is whether eDNA can be differentiated between different life-history stages of the same species. Answering these questions would significantly add to the value of eDNA as a field survey tool. Still even with limitations to using eDNA for biomass and aging estimates in the field, there are multiple ways in which eDNA can and is used to support fisheries and aquaculture.

Challenges of Interpreting eDNA Data

As illustrated in **Fig. 1**, the quantity of eDNA in seawater is affected by species properties including size, age, behavior such as spawning; as well as environmental parameters such as temperature, salinity, and UV light. Understanding the processes affecting your sample is critical to interpreting your data. Further, being aware of the biases inherent in the amplification process, *i.e.* some genetic sequences amplify more efficiently than others, is important (Kelly *et al.*, 2019). The extent to which these uncertainties are significant for your research depends much upon temporal and spatial scaling

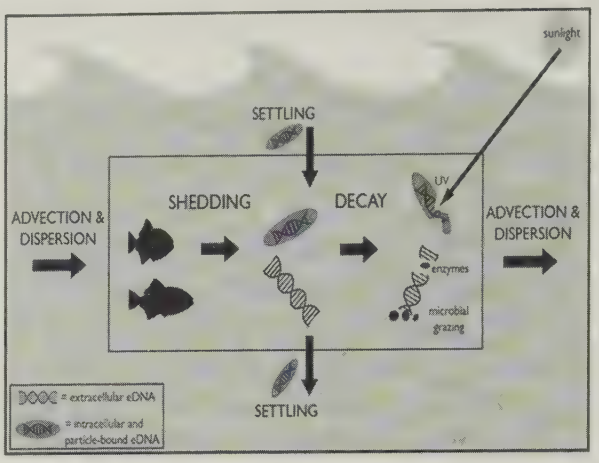

Fig. 1. Conceptual model of eDNA distribution in the water column. From Sassoubre *et al.* (2016).

requirements. Whether a decay rate of hake DNA is 2 days or 4 days is probably not significant for assessing distribution in the middle of the Pacific Ocean, but that same difference can be extremely important for estimating distribution of Atlantic salmon in a river in Maine or distribution of aquaculture escapees in an embayment.

Conceptually, to fully understand eDNA data from the field surveys, one would need to account for all the processes affecting eDNA distribution as shown **Fig. 1**, including shedding, enzymatic and other types of degradation, and physical processes such as advection, dispersion and export for example as feces. Realistically, we are likely to make faster progress in using eDNA as a survey tool by comparing field eDNA with trawl and acoustic survey data, and using the laboratory experiments to help in interpretation.

Ecosystem Services

Environmental DNA is often used to identify presence or absence of species. This can be *e.g.* an indicator of ecosystems services such as prey species' refugia or the impact of aquaculture on finfish diversity. In Milford, CT, USA, a team of researchers from the NOAA Milford laboratory has been measuring eDNA along with use of underwater video to evaluate the effect of caged oyster on fish diversity (Liu *et al.*, 2019). In the first year of investigation they identified 23 species from a total of 49 samples or 20 million reads from

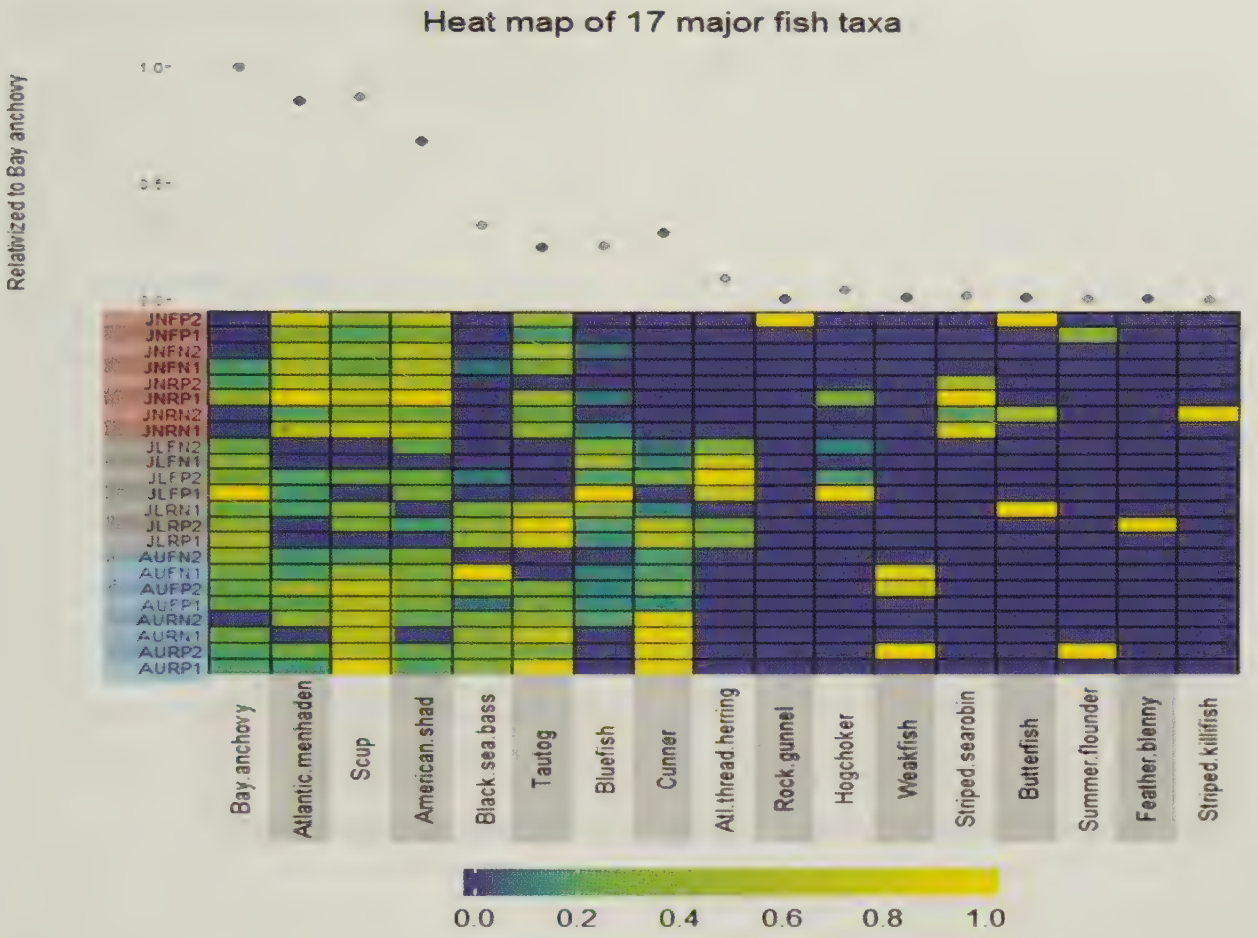

Fig. 2. Environmental DNA as a tool to assess spatiotemporal finfish distribution in relation to shellfish aquaculture operations. From Liu *et al.* (2019).

the sequencing data (**Fig. 2**). Notably, the species identified by eDNA overlapped but were not identical to the species identified with video, thereby leading researchers to conclude that employing both methods concurrently would give a more comprehensive result.

When is Quantity Important?

Of course scientists often want to know more than presence – absence of species in the field, and being able to estimate quantity even if in relative terms can be important. This is true, for example, for detection of pathogens in relation to critical thresholds for human consumption or permissible transport, estimating the frequency and magnitude of encounters of protected species with aquaculture gear, estimating the effect of aquaculture on the food web, or estimating the effect of aquaculture on benthic community composition. What is necessary to make eDNA a more useful quantitative survey tool?

As illustrated in **Fig. 1**, the quantity of eDNA in seawater is affected by species-specific rates of metabolism and physiology as well as environmental parameters. In collaboration with the Cold Spring Harbor Laboratory, University of Buffalo, Stanford and Monmouth Universities, NOAA scientists conducted laboratory experiments in a closed recirculating system at Sandy Hook, NJ, USA to determine species-specific shedding and decay rates of eDNA under different environmental conditions. To date we have run trials with adult black sea bass, juvenile winter flounder, and currently adult summer flounder. Some results from the black sea bass run in **Fig. 3** show eDNA in equilibrium and eDNA degradation. Most eDNA degraded within about two days.

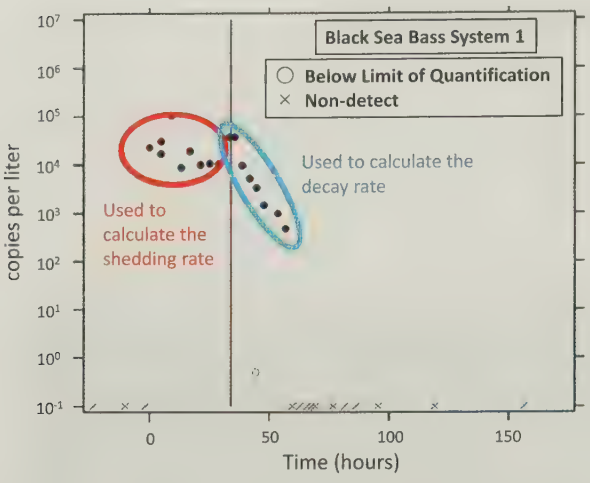

Fig. 3. Quantification of Environmental DNA shedding and decay rates for black sea bass. From Kirtane *et al.* (in preparation).

To apply such shedding and degradation rates to field investigations, Sandy Hook staff conducted sampling on cruises with the Marine Academy of Science & Technology (MAST) in NJ in early May, 2019, as well as on the Northeast Fisheries Science Center fall 2019 bottom trawl survey. On both cruises, water was filtered for later analysis; results are pending and should be available as the US response to the current pandemic permits.

One of the important next steps to making this a quantitative survey tool is to develop eDNA particle transport models, as has been done off Southern California. These models are intended to indicate the geographical origin of collected eDNA (Andruszkiewicz *et al.*, 2017). In addition to supporting stock assessments, this approach can be useful to tracking the distribution and abundance of *e.g.* escapees from penned cultured salmon and other species.

Notably, a promising application of environmental DNA in field surveys will be to pair this approach with acoustic surveys. This is particularly attractive because it would be relatively easy to develop a strategy for rapid sampling and rapid analysis, using eDNA species data to ground truth acoustic data. The development of towed midwater eDNA sampling equipment will significantly facilitate this methodology; several efforts are currently underway to develop this equipment. For example NOAA staff in Sandy Hook, NJ have partnered with MAST to construct a midwater eDNA sampler for deployment from small vessels.

Environmental DNA can be an important tool for benthic surveillance to assess habitat preferences (Takahara *et al.*, 2019) as well as impacts of caged cultured fish on sediments and associated biota. There are clear correlations reported for changes in OTUs and species as measured using eDNA in relation to input of organic waste, *e.g.* from aquaculture, to sediments (Keeley *et al.*, 2019). This has also been explicitly demonstrated for penned salmon (Dowle *et al.*, 2015). Notably even if species cannot be identified from the OTUs due to lack of appropriate reference libraries, the OTU response to a disturbance reflects a biological impact and can be analyzed without species identification. Thus eDNA is a promising tool to quickly measure biological impacts in sediments due to aquaculture and can be an important method to help identify Aquaculture Opportunity Areas and to monitor operations impacts.

It is also important to note that disease is one of the greatest impediments to successful aquaculture operations. Tracking and modeling the spread of disease will be key to managing the epidemiology of viruses, bacteria and parasites on cultured shellfish and finfish. eDNA is a tool which can be used for pathogen surveillance in the field. In conjunction with environmental and hydrographic information, this will assist modelers to predict rates and areas of spread of specific diseases. Notably, this also allow forecasts in relation to changing climate. Further, the potential is large for application of eDNA for seafood inspection, measuring not only fish and shellfish pathogens but also human pathogens such as Vibrio.

Aquaculture Operations Conditions and Fish Health

eDNA is potentially a valuable tool for studying health-related effects of aquaculture operations including use of pharmaceuticals and other chemicals on cultured fish and shellfish. Microbial diversity of gut microflora has been studied in various fish species collected from Japan's coastal waters using next-generation sequencing. In one study metabolites and bacterial eDNA in feces were

analyzed as indicators for fish health. The potential of this approach as a non-invasive inspection technique in aquaculture was suggested by Asakura *et al.* (2014).

Protected Species

In the USA, one of the greatest concerns impeding the permitting of offshore aquaculture is the risk of entanglement of protected species such as the right whale in the NE USA. To date, predicting species distributions and migrations have largely relied on models of food supply availability and habitat suitability of the species of interest such as right whales. Use of environmental DNA as a surveillance tool for protected species may enable us to go from modeling their distribution to actual observation for assessing encounter rates of aquaculture gear with target species. With the use of in situ deployed automated samplers and sequencers (*e.g.* from Monterrey Bay Aquarium and Research Institute) eDNA is a promising tool to actually record the presence of protected species or their prey in the direct vicinity of aquaculture sites. Getting more field observation data will be pivotal to informing and verifying the reliability of entanglement models.

Summary

Sampling and processing of environmental DNA samples are relatively rapid and inexpensive. eDNA data can support multiple applications to support aquaculture. The applications can be qualitative and quantitative. Applications are useful to describe species diversity; to monitor for finfish escapees, pathogens and protected species; to assess impact of aquaculture operations on habitats; to evaluate suitability of habitats for cultured shellfish; to assess the health of cultured species; and other applications.

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A key publication on experiments for shedding and decay rates of eDNA from marine finfish. Also includes a much cited conceptual model for processes in the field affecting concentrations of eDNA.

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Quantification of Environmental DNA (eDNA) Shedding and Decay Rates for Three Marine Fish in Support of Quantitative Field Investigations. Describes the results from laboratory experiments on one pelagic fish species and two flatfish. Also relates these data to field surveys for these species with comparisons to trawl data.

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A stable isotopic approach to investigate nitrogen pathways in a coastal aquaculture area

Satoshi WATANABE*

Abstract: Coastal eutrophication and consequent environmental deterioration have been problematic in many parts of the world. However, oligotrophication and resulting reduction in coastal fisheries and aquaculture productivity have also been a recognized problem in some parts of Japan. Some studies argue that declining fisheries production of coastal resources and unfed aquaculture production is partially attributable to the reduced nutrient concentrations in the coastal waters in recent years. Eutrophication mitigation efforts have reduced the terrestrial nutrient load to the coastal environment over the past forty years in Japan. Excessive oligotrophication has allegedly reduced primary productivity and hence carrying capacity of coastal ecosystems supporting coastal fisheries and aquaculture. Fisheries production of the Manila clam, *Ruditapes philippinarum*, has decreased by 95% over the past three decades. Insufficient food supply is one amongst many speculated factors causing the reduction. However, complex nutrient flow in coastal environments has not necessarily been elucidated. Our stable isotopic ($\delta^{13}C$ and $\delta^{15}N$) studies have suggested that there are large and small scale variations in the nutrient pathways from inorganic nutrients to the clam. Clams inhabiting the same tidal flat only 10 m apart from each other can have a different food environment as indicated by $\delta^{13}C$. Pervasive effects of terrestrial nutrient load on the food availability of the clam may be ascertained by $\delta^{15}N$. Stable isotopic methods may also be used to determine nutrient flow within an integrated multi-trophic aquaculture (IMTA) system, which has potential to enhance unfed aquaculture production in an oligotrophic environment.

Key words: eutrophication, oligotrophication, stable isotope ratio, nitrogen, carbon, IMTA

Introduction

Coastal eutrophication due to increasing anthropogenic activities and consequent environmental deterioration, such as anoxia, harmful algal blooms and hydrogen sulfide emission have been problematic in many parts of the world, sporadically causing mass mortality of aquatic organisms. While this holds true for Japan, oligotrophication and resulting reduction in coastal fisheries and unfed-aquaculture productivity have also been a recognized problem in some parts of Japan. Some studies argue that ever-dwindling fisheries production of coastal resources, as well as unfed aquaculture production of inorganic and organic extractive species is partially attributable to the reduced nutrient levels in the coastal waters in recent years (Tanda and Harada, 2012; Yamamoto, 2003).

Eutrophication mitigation efforts have reduced the load of nitrogen and phosphorus of terrestrial and aquaculture origins to the coastal environment over the past forty years in Japan. Allegedly excessive oligotrophication has reduced primary productivity and hence carrying capacity or coastal ecosystems supporting coastal fisheries and aquaculture. However, complex nutrient flows within coastal ecosystems are not necessarily well understood.

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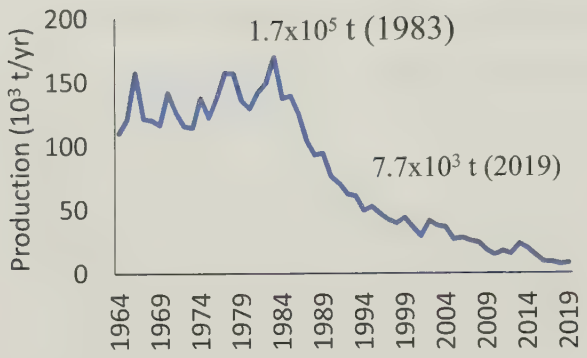

Fig. 1. Manila clam fishery production in Japan.

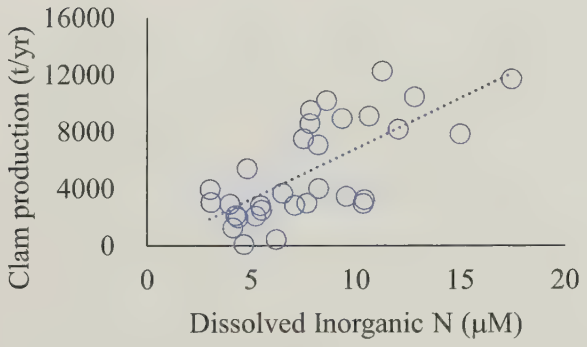

Fig. 2. Relationship between the annual mean dissolved inorganic nitrogen level and Manila clam fisheries production in Ise, Bay, Mie Prefecture from 1985 to 2015.

This paper describes a stable isotopic approach to investigate nutrient pathways in coastal aquaculture areas, with Manila clam, *Ruditapes philippinarum*, and Integrated Multi-Trophic Aquaculture (IMTA) as examples.

Manila Clam Fisheries Production in Japan

Manila clam is one of the iconic coastal species facing serious stock depletion in Japan. National fisheries production, inclusive of bottom culture, of the Manila clam has decreased by 95% over the past three decades (**Fig. 1**) (e-Stat). The production reached the peak at 169,621 t in 1983, and it gradually and continuously decreased to 7,736 t in 2018. Japan used to be the largest producer of the clam in the world, but it imported 42,482 t of the clam, amounting to JPY 8.6 billion from countries such as China and South Korea in 2017 (Ministry of Finance, Trade statistics of Japan).

Although the mechanism of the clam production decline is not necessarily elucidated, insufficient food supply associated with recent coastal oligotrophication is one amongst many speculated factors causing the reduction. The relationship between the annual mean dissolved inorganic nitrogen (DIN) level (Mie Prefecture) and the Manila clam fisheries production in Ise Bay from 1985 to 2015 (e-Stat) showed a significant negative correlation (**Fig. 2**) (r^2=0.49, $p < 0.001$, N=30). Like many other bivalves, the Manila clam is thought to filter-feed microalgae. Reduced nutrient level in coastal waters is thought to diminish primary productivity, thereby reducing the carrying capacity of the area. However, this hypothesis has not been supported yet because of the complexity of nutrient cycles in coastal areas.

Small Scale Variation in Food Items of the Clam

Food availability to the clam is often represented by chlorophyll *a* levels in a water column. In many studies, however, researchers take water samples from water columns far above the bottom water that the clams inhale and filter-feed. Collection of bottom water within a few centimeters from the bottom is laborious, and water parameters measured for other general purposes are often used to ascertain the food availability. Also, information on the availability and nutritional quality of different microalgal species is limited. These issues make accurate determination of food availability and nutrient flow within the food chain difficult.

Stable isotope ratios of carbon (δ^{13}C) and nitrogen (δ^{15}N) are widely used in the studies of interactions of food webs in aquatic ecosystems. Our stable isotopic study (Watanabe *et al.*, 2009a) has suggested that the food source of the clam may differ within a small area depending on the bottom topography of the habitat.

Many filter feeding bivalves are generally held to be phytoplanktivorous; however, they also feed on benthic microalgae. Provisional ratio of the planktonic (*i.e.* pelagic) to benthic microalgae seems to be affected by the extent of resuspension of the latter from the sediment to the bottom water to make them available to the clam. In our study

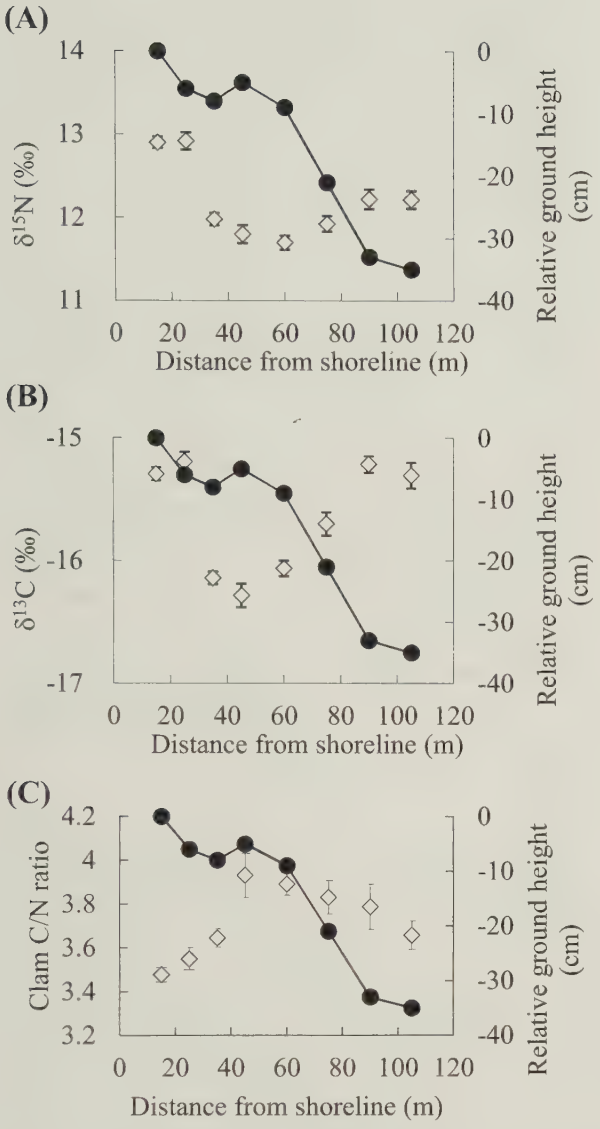

Fig. 3. δ^{15}N (A), δ^{13}C (B) and C/N ratio (C) of whole soft tissue of Manila clam at eight stations on an inshore – offshore transect in tidal flat in Yokohama Marine Park (after Watanabe *et al.*, 2009a).

of δ^{13}C and δ^{15}N of the clams collected along a inshore-offshore transect in an artificial tidal flat in Yokohama Marine Park, clams inhabiting the same tidal flat only 10 m apart from each other had a different δ^{13}C and δ^{15}N signature, as well as carbon to nitrogen ratio (C/N) depending on the position on the sandwave (**Fig. 3**). This indicates that the clam assimilates different food items depending on the position on the sandwave. In general, benthic microalgae tend to have higher δ^{13}C than do phytoplankton, and higher C/N indicates higher glycogen (*i.e.* energy reserve for bivalves) content of the clam. Resuspension of benthic microalgae may be more active near a dune presumably because of higher wave action, increasing the availability of benthic microalgae to the clam. The clams utilizing both planktonic and benthic microalgae near the dune had higher δ^{13}C, and they seemed to have better nutritional condition as indicated by higher C/N.

Benthic microalgae are important food source for the Manila clam, and chlorophyll *a* concentration in the bottom water should be analyzed to determine food availability to the clam.

Large Scale Variation in Nutrient Pathway to the Clam

Anthropogenic nitrogen is known to have a higher nitrogen stable isotopic (δ^{15}N) signature than nitrogen from atmospheric deposition (2‰ – 8‰) and nitrogen fixed by cyanobacteria (−2‰ – 0‰) (McClelland *et al.*, 1997; Oowada *et al.*, 2003). Treated water of sewage, for example, contains DIN with higher δ^{15}N elevated by denitrification during the treatments (Macko and Ostrom, 1994). Agricultural fertilizer also enhances soil denitrification and increases δ^{15}N in groundwater (Ogawa *et al.*, 2001). Thus, δ^{15}N in DIN acts as an indicator of the level of anthropogenic nitrogen loads to coastal waters.

The δ^{15}N in the soft tissues of the Manila clam was found to be positively correlated to the total DIN (*i.e.* sum of nitrate, nitrite and ammonium nitrogen) concentration in the bottom water in tidal flats in Kanagawa, Shizuoka and Fukuoka Prefectures with different eutrophication levels (Watanabe *et al.*, 2009b). This indicates that the clam δ^{15}N can be a proxy for the anthropogenic nitrogen load to the coastal environment.

The concentration and δ^{15}N of the total DIN were positively correlated with each other in the studied areas (**Fig. 4**), indicating that input of terrestrial nitrogen with higher δ^{15}N elevates the DIN level in coastal waters. The δ^{15}N of the clam soft tissue, particulate organic matter (POM) in seawater and sediment organic matter (SOM) in tidal flat surface were higher in areas with higher total DIN concentration of bottom water (**Fig. 5**). The higher δ^{15}N in the clam was considered to be attributable to higher δ^{15}N in the food particles (POM and SOM),

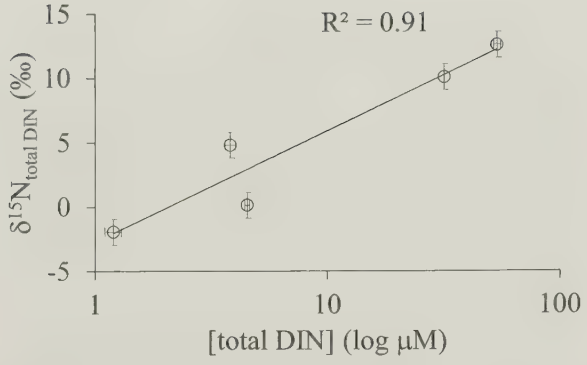

Fig. 4. Relationship between the mean concentration and mean $\delta^{15}N$ in total DIN in bottom water collected from different tidal flats in Japan (after Watanabe *et al.*, 2009b).

especially those in SOM.

This study demonstrates that elevated $\delta^{15}N$ in DIN in coastal waters due to anthropogenic nitrogen loads is reflected in the $\delta^{15}N$ in the clam. Thus, pervasive effects of terrestrial nutrient load to the food availability to the clam may be ascertained by the clam $\delta^{15}N$. This can be a powerful tool to understand the effects of eutrophication and oligotrophication of coastal waters to the clam fisheries productivity.

Nutrient Flow within Integrated Multi-Trophic Aquaculture

Integrated multi-trophic aquaculture (IMTA) is a technique to use unfed aquaculture of inorganic and organic extractive species (*e.g.* algae and macrobenthos) to consume effluent from fed aquaculture of finfish (Chopin, 2006). Although IMTA is usually proposed as a mitigation measure for eutrophication by intensive fed aquaculture, it has a potential to alternatively enhance unfed aquaculture production in an oligotrophic environment. In either case, nutrient tracing is important to design an efficient IMTA system.

In order to trace nutrient flow within an IMTA system using stable isotopic method, not only is it necessary to determine isotopic fractionation between organic extractive species (*e.g.* sea cucumber, Watanabe *et al.*, 2013) and food, but to have information on how stable isotopic signatures differ between the aquaculture feed and fish feces,

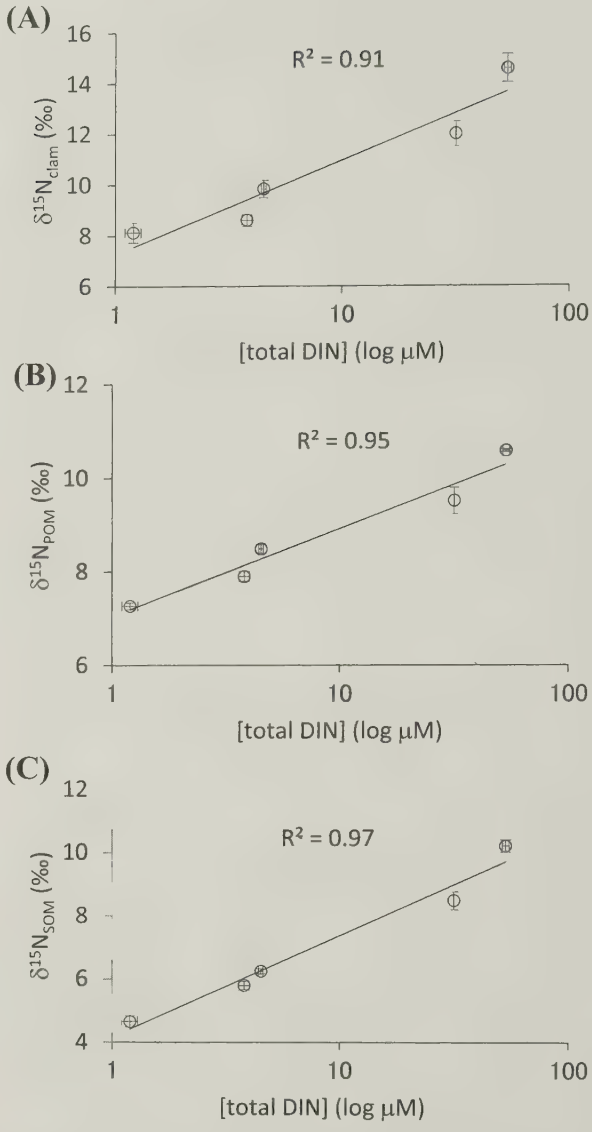

Fig. 5. Relationship between the mean concentration (± SE) of the total DIN and $\delta^{15}N$ in Manila clam (A), POM in the bottom water (B) and SOM in the tidal flat surface (C) (after Watanabe *et al.*, 2009b).

both of which can be a nutrient source for the organic extractive species.

We compared the $\delta^{13}C$ and $\delta^{15}N$ of feces collected from five different finfish species (Asian seabass, mangrove red snapper, milkfish, snubnose pompano and orange-spotted spinefoot) commonly produced in aquaculture in Southeast Asia (Watanabe unpubl. data). We fed the same formulated feed to these fish to determine difference in $\delta^{15}N$, $\delta^{13}C$, and contents of nitrogen and carbon between the feed and feces. We found that while the $\delta^{15}N$ of feces showed no significant difference from that of

the feed among all the species, $\delta^{13}C$ and C/N ratio were different among the species. Therefore, $\delta^{15}N$ can be used to determine the contribution of the nitrogen from fed aquaculture to growth of organic extractive species, but it cannot determine whether the nitrogen was used by the organic extractive species as leftover feed or fish feces.

In order to using $\delta^{13}C$ to trace carbon flow within an IMTA system, the relationship of $\delta^{13}C$ between feed and fish feces must be determined beforehand for each fish species and feed. The difference we observed in $\delta^{13}C$ and C/N ratio may be attributable to different digestibility of each fish species. Formulated feed usually contains plant ingredients such as wheat flour and rice bran as a binder. Herbivorous species (orange-spotted spinefoot) may be able to digest and assimilate more plant ingredients (*i.e.* rich in carbon) than do carnivorous species (Asian seabass).

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Annotated Bibliography of Key Works

(1) Watanabe S., Kodama M., Sumbing J. G., and Lebata-Ramos M. J. H., 2013: Diet-tissue stable isotopic fractionation of tropical sea cucumber, *Holothuria scabra*. *JARQ*, **47**, 127–134.

In order to provide a basis for stable carbon and nitrogen isotope ratio ($\delta^{13}C$ / $\delta^{15}N$) analysis that will allow determination of assimilated organic matter in the sea cucumber, *Holothuria scabra*, diet-tissue fractionations were experimentally determined by mono-feeding rearing with diatoms. While $\delta^{15}N$ fractionation of the whole body wall (2.4‰) was similar to the commonly accepted value (2.6–4‰),

$\delta^{13}C$ fractionation of the body wall (4.2‰) showed considerable discrepancy with the commonly accepted value (0–1‰) due to the high content (35% dry wt/wt) of calcareous spicules ($CaCO_3$) in the body wall, which had significantly higher $\delta^{13}C$ (−8.6‰) than the organic fractions. Computational elimination of spicules based upon spicule content and spicule $\delta^{13}C$ reduced the $\delta^{13}C$ fractionation of the body wall to 1.5‰, close to the common value. $\delta^{13}C$ fractionation after spicule removal by acid decarbonation and subsequent rinsing (3.2‰) did not agree with the common value, and $\delta^{15}N$ fractionation was significantly elevated by decarbonation. $\delta^{15}N$ and $\delta^{13}C$ fractionations of the intestine (1.5 and 2.2‰, respectively) did not agree with the common values. Since $\delta^{13}C$ and $\delta^{15}N$ of the feces did not differ significantly from those of the diet, feces may be used to determine ingested organic matter in the wild.

(2) Watanabe S., Kodama M., Zarate J. M., Lebata-Ramos M. J. H., and Nievales M. F. J., 2012: Ability of sandfish (*Holothuria scabra*) to utilise organic matter in black tiger shrimp ponds. *ACIAR Proceedings,* **136**, 113–120.

Due to frequent viral disease outbreaks, a large proportion of shrimp aquaculture in South-East Asian countries has switched from black tiger shrimp (*Penaeus monodon*) to *P. vannamei*, an exotic species originally imported from Latin America. One of the causes of disease outbreaks is thought to be poor water and sediment conditions in the shrimp ponds, which may aggravate disease symptoms. To obtain basic information for co-culture methods of black tiger shrimp and sandfish (*Holothuria scabra*) for possible mitigation of shrimp-pond eutrophication and prevention of disease outbreaks, basic laboratory experiments were conducted. A feeding trial of juvenile sandfish showed that they do not grow well with fresh shrimp feed on hard substrate. Another trial indicated that sand substrate enhances the growth of juvenile sandfish fed with shrimp feed. A feeding trial using shrimp tank detritus, shrimp feces and *Navicula ramosissima* (a benthic diatom) as food sources showed that sandfish grew fastest with the feces, followed by detritus and *N. ramosissima*. Dissolved oxygen consumption and acid-volatile sulfur levels in the shrimp tank detritus were reduced by sandfish feeding. This suggests that sandfish are capable of growing with organic matter in shrimp ponds and can bioremediate shrimp-pond sediment.

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Immunological assays of hemocytes in molluscan bivalves as biomarkers to evaluate stresses for aquaculture

Huiping YANG*

Abstract: As an important aquaculture section, molluscan aquaculture has traditionally accounted for about 60% of total marine aquaculture production worldwide. The molluscan aquaculture species are majorly bivalves, including oysters, clams, scallops, and mussels. Challenges in molluscan aquaculture include diseases, environmental stresses, coastal pollutions, and seed quality and quantity. This mini review summarized current research updates on immunological assays of hemocytes of molluscan bivalves against the biotic (*e.g.* bacteria, viruses, or protozoan parasites) and abiotic stresses (*e.g.* temperature shock, fluctuated salinity, or environmental toxins). As the frontline of immune system, hemocytes play a significant role against these stresses. The immunological assays of hemocytes could be used as effective biomarkers to evaluate the effects of biotic and abiotic stresses in aquaculture operation and breeding programs.

Key words: molluscan bivalves, immunological assays, hemocyte, stress, biomarker

Introduction

Molluscan aquaculture is a US$23.9 billion industry worldwide for seafood production (FAO, 2018). Sustainability of molluscan aquaculture industry faces many challenges including seed quality and quantity, environmental stresses and climate changes, diseases, natural and genetic resources, best practices of operations, and regulatory scrutiny (Dumbauld *et al.*, 2009). Accordingly, efforts have been made on overcome these challenges through genetic breeding for stock enhancement (Hulata, 2001), disease diagnosis and control by use of probiotics (Hoseinifar *et al.*, 2018), natural resources conservation management (Beck *et al.*, 2011), improvement of water quality through land use management, employment of best practices, and extensive education programs.

Immune system in molluscan bivalves was firstly reported in *Mytilus californianus* about hemolymph agglutinins (Tyler, 1946), which were observed later in butter clams, *Saxidomus giganteus* (Johnson, 1964), and eastern oysters, *Crassostrea virginica* (Tripp, 1966). Hemocytes in bivalves have been studied extensively since the 1970s and summarized in two review publications (Hine, 1999; Anisimova, 2013). In recent years, the molecular mechanisms and signal pathways of bivalve immune system have been becoming research focus to understand the immune protective strategies from various pathogens and environmental stresses (Song *et al.*, 2010).

This mini review summarized the immunological assays of hemocytes from aquaculture molluscan bivalves in response to biotic and abiotic factors, and it is expected that these hemocyte assays could be used as effective biomarkers for disease diagnosis, evaluation of environmental stresses, and breeding tools.

Immune System in Molluscan Bivalves

Immune system in vertebrates includes a series of collectively effective defenses against diseases and pathogen invasions. The first defense is the ***physical barriers***, such as the skin, which can prevent from colonization with other organisms and move

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inhaled materials using the mucociliary apparatus (ciliated epithelial cells and mucus-secreting cells). The second defense is ***innate immunity*** which is a primitive nonspecific immunity to against any pathogens that enter the body rather than targeted specific invaders. The third defense is a complex, specific, and long-lasting ***adaptive immunity*** which relies on the accumulated memory cells after exposure to pathogens.

Molluscan bivalves possess an open circulatory system. Bivalves pump hemolymph into the open body cavity (hemocoel), circulates in hemolymph vessels and sinuses as well as throughout soft tissues, thus the hemolymph can bath the internal organs and deliver nutrients and gases. It is believed that bivalves use their hemocytes and humoral proteins in circulatory system to provide internal defensive functions against various pathogen and environmental stresses (Bayne, 1983).

1. Physical barriers

The external shells in molluscan bivalves are the most important physical barrier to protect their soft tissues and organs and prevent from predators, parasites, harmful substances, and environmental changes. For example, the northern quahog (also called hard clam), *Mercenaria mercenaria*, can keep their shells closed without any movement and ejection for days at −1.0 to 1.9℃ seawater (Loosanoff, 1939); the Pacific oyster, *Crassostrea gigas*, can close their shells tightly at 4℃ air dry for 47.8 days (50% lethal time) (Kawabe *et al.*, 2010); scallops can swim away by flapping their shells to escape predators or environmental stresses. Additionally, bivalves can use their gills and labial palps to select food particles and wrap un-selected particles with mucus for ejection (Shumway *et al.*, 1985; Ward and Shumway, 2004).

2. Innate immunity

The innate immune in molluscan bivalves is believed to achieve through humoral innate immunity, which involves in molecules (*e.g.* proteins) in the body humors to stop the growth of pathogens or clump them together, and cellular immunity of hemocytes, which involves in phagocytes to ingest and degrade pathogens (Bayne, 1983).

Humoral immunity can be achieved through antimicrobial peptides, which is an evolutionarily conserved component of innate immunity in all classes of life and represent the main form of invertebrate systemic immunity. In bivalves, the identified humoral factors together with their immune functions included atrial natriuretic peptides (ANPs) in hemolymph and heart of the eastern oyster, *Crassostrea virginica* (Vesely *et al.*, 1993), catecholamines in the giant scallop, *Placopecten magellanicus* (Pani and Croll, 2000), lectins in the pearl oyster, *Pinctada maxima* (Flower *et al.*, 1985), and the giant clam, *Hippopus hippopus* (Puanglarp *et al.*, 1995), and hemagglutinins in the eastern oyster (Li and Flemming, 1967) and the northern quahog (Tripp, 1992). The profile of total protein, ions, and sugars composition in hemolymph have been documented in *Mya arenaria* and connected with their immune functions (Sunila and Dungan, 1992; Rees *et al.*, 1993).

Hemocyte immunity is the fundamental immune feature and usually achieved by recognition of foreign substances and subsequent ingestion (Pila *et al.*, 2016). The immunological assays of hemocyte in molluscan bivalves were stated as follows in next Section.

3. Adaptive immunity (also called acquired immunity)

Adaptive immunity is a more sophisticated system to recognize and destroy specific invaders based on cellular memory. The process of this defensive reaction normally uses specific antigens which are activated by exposure to pathogens. Therefore, it is antigen-specific functions through cell-mediated system. Adaptive immunity system uses an immunologic memory to learn about the pathogen and enhance the immune response, accordingly. This system is more effectively and specifically to the pathogens, but usually much slower to respond to threats and infections than the innate immunity.

Generally, adaptive immunity is considered to exist only in vertebrates. However, in recent years, adaptive immunity has been identified in invertebrates and even bacteria, such as the CRISPR/cas9 system which can recognize and destroy the invaded virus RNA sequence (Zhang *et*

al., 2012). For bivalves, the first evidence of antiviral immune priming was just reported in the Pacific oyster (Lafont *et al.*, 2020) against the herpes-like virus Ostreid herpesvirus 1, a major viral disease triggers the Pacific oyster mortality syndrome (Segarra *et al.*, 2010). The injection of various nucleic acids showed the capability to trigger oysters to protect them against a subsequent viral infection. Additionally, specific genes in adaptive immunity pathway in abalones were found to be up/down regulated when exposure to thermal shock and/or hypoxia (He *et al.*, 2017; Zhang *et al.*, 2019) and in the Pacific oyster when exposure to environmental stresses (Guo *et al.*, 2015).

Immunological Assays of Hemocytes in Molluscan Bivalves

Hemocytes in bivalves have been reported to participate a variety of physiological and immune functions, such as wound repair, shell formation and healing, nutrient transport and digestion, excretion, and internal defense (Anderson and Good, 1976; Song *et al.*, 2010; Pila *et al.*, 2016). When exposure to invasive pathogens, hemocytes can encapsulate them and subsequently destroy them via enzyme activity and oxygen metabolite release defense the invading microbes (Song *et al.*, 2010). A comprehensive review has summarized the morphology and functionality of hemocytes in bivalves for further reading (Anisimova, 2013).

In this publication, a literature search was performed about bivalve hemocyte functions under different stresses (**Table 1**). Briefly, the hemocyte assays in bivalves are as follows.

1. Hemocyte morphology and cell types

Based on the morphological characteristics such as cell sizes and cytoplasmic inclusions, the hemocytes in bivalves are classified into two types – granulocyte and hyalinocyte (agranulocyte). For some species, a third type of hemocytes with different characteristics was reported with different names. So far, two reviews have made comprehensive summaries on bivalve hemocyte cells types for further reading (Hine, 1999; Anisimova, 2013). The methodologies for hemocyte morphological observation include light microscopy, transmission electron microscopy, flow cytometry, and monoclonal antibody (Noël *et al.*, 1994).

(1) Granulocytes

Granulocytes were found to be the major hemocyte type in bivalves. Granulocytes were usually characterized with cytoplasmic granules and have a low nucleus: cytoplasm ratio. Depending on the granular features granulocytes can be sub-categorized as eosinophilic granulocytes which contain cytoplasmic large eosinophilic granules and stain with acid stains (such as eosin with pink color), basophilic granulocytes which contain small granules and stain with alkaline stains (such as methylene blue with blue color). In several studies, granulocytes were also divided and named as small and large granulocytes. The nuclei of granulocytes are usually uninucleate or binucleate with eccentric, spherical, or occasionally ovoid morphology and stains as dark blue with DNA staining by Giemsa.

(2) Agranulocytes (Hyalinocytes)

Agranulocytes were also named as hyalinocytes in many publications based on microscopic observations. As the name reflects, these cells are characterized with few or without visible cytoplasmic granules in cytoplasm and have relative larger nucleus. Based on the cell size, agranulocytes can be classified into large hyalinocytes (agranulocytes) and small hyalinocytes (agranulocytes, also called blast-like cells in several publications) with a central ovoid or spherical nucleus surrounded by a rim of scant cytoplasm lacking organelles (Bachère *et al.*, 1988).

(3) Other types

Besides the granulocyte and agranulocyte types, other hemocyte types were observed in bivalve species. For example, cells had the general appearance of granulocytes, low nucleus: cytoplasmic ratio and round nucleus, but had few or no granules. These cells were regarded as fibrocytes in the eastern oyster and the northern quahog (Foley and Cheng, 1972) and later were considered as degranulated granulocytes following phagocytosis (Mohandas and Cheng, 1985). Depending on cell characterizations, different names have been used to describe these hemocytes by different authors with no systematic rules (Hine, 1999).

Table 1. The immunological assays of hemocytes in molluscan bivalves under challenges of environmental and biotic stresses

SPECIES	STRESS*	IMMUNOLOGICAL ASSAYS OF HEMOCYTES**	REFERENCE
Crassostrea gigas	Spatial and temporal	Hemolymph microbiota status	(Lokmer *et al.*, 2016)
Crassostrea gigas	T and infection	Hemolymph microbiota status	(Lokmer and Wegner, 2015)
Crassostrea gigas	Seasonal changes	Persistence, seasonal dynamics, and pathogenic potential of *Vibrio*	(Wendling *et al.*, 2014)
Crassostrea gigas	Herpesvirus	Antiviral activity of hemolymph protein against herpesviruses	(Green *et al.*, 2014)
Crassostrea gigas	Hypoxia	Regulation of an isoform of AMP-activated protein kinase	(Guevelou *et al.*, 2013)
Crassostrea gigas	Bacteria	Antimicrobial peptides analysis in hemolymph	(Defer *et al.*, 2013)
Crassostrea gigas	Air dry, hypoxia	Changes of pO_2, pCO_2, pH, and osmolality in hemolymph	(Kawabe *et al.*, 2010)
Crassostrea gigas	Virus infection	Antiviral activity in hemolymph	(Olicard *et al.*, 2005)
Crassostrea gigas	Disturbances, salinity, and T	Catecholamine changes in the hemolymph	(Lacoste *et al.*, 2001)
Crassostrea gigas	T, TBT, and trace metals	The alteration of spontaneous hemocyte aggregation	(Auffret and Oubella, 1997)
Crassostrea gigas	Repeated bleeding	THC, phagocytosis, hemolymph pH, and pO_2	(Jones *et al.*, 1993)
Crassostrea gigas	*Vibrio anguillarum*	Agglutination activity after challenge to *Vibrio*	(Olafsen *et al.*, 1992)
Crassostrea gigas	Virus – T3-coliphage	Hemocyte neutralizing activity	(Bachere *et al.*, 1990)
Crassostrea madrasensis	*Vibrio alginolyticus*	THC, serum protein, lysozyme activity	(Ittoop *et al.*, 2010)
Crassostrea rhizophorae	Seasonal change	Carbonic anhydrase to reflect environmental contaminations	(dos Santos *et al.*, 2017)
Crassostrea rhizophorae	Virus infection	Evaluation of antiviral activity in hemolymph	(Carriel-Gomes *et al.*, 2006)
Crassostrea virginica	Cadmium	Apoptosis	(Sokolova *et al.*, 2004)
Crassostrea virginica	Heat shock to 28℃	Changes of hemocyte cell types and viability	(Hegaret *et al.*, 2003)
Crassostrea virginica	*Perkinsus marinus*	Function of hemocytes to kill *Perkinsus marinus*	(Volety and Fisher, 2000)
Crassostrea virginica	Fungicide	Phagocytosis, reduced pyridine nucleotides, and ROS	(Baier-Anderson and Anderson, 2000)
Crassostrea virginica	Salinity	Atrial natriuretic peptides (ANPs) in the circulation hemolymph	(Palmer *et al.*, 1994)
Crassostrea virginica	T, salinity, food, *Dermo*	Hemolymph lysozyme activity and protein profile	(Chu and Lapeyre, 1989)
Crassostrea virginica	TBT	Kinetic regulation of chloride-ion and osmotic-pressure in hemolymph	(Bokman and Laughlin, 1989)
Crassostrea virginica	Repeated bleeding	Hemolymph protein and parasitism infection of *Haplosporidium nelsoni*	(Ford, 1986b)
Crassostrea virginica	*Haplosporidium nelsoni*	Hemolymph proteins from resistant and susceptible oysters	(Ford, 1986a)
Crassostrea virginica	Starvation, salinity, T	Protein and carbohydrate levels in the hemolymph	(Fisher and Newell, 1986)
Crassostrea virginica	Cercariae	Encapsulation of cercariae by hemocytes	(Font, 1980)
Crassostrea virginica	Bacteria	Clearance of enteric bacteria from the hemolymph	(Hartland and Timoney, 1979)
Crassostrea virginica	*Bacillus megaterium*	Aminopeptidase activity in hemocytes and hemolymph	(Yoshino and Cheng, 1976)
Crassostrea virginica	*Minchinia nelsoni* (MSX)	Hemolymph enzyme activities during oyster-MSX interaction	(Douglass and Haskin, 1976)
Crassostrea virginica	*Micrococcus lysodeikticus*	Hemocyte lysozyme capability	(Mcdade and Tripp, 1967)
Ostrea edulis	*Bonamia ostreae*	Apoptosis of hemocytes	(Gervais *et al.*, 2018)
Ostrea edulis	Salinity, T	Expression of HSP/C70 or metallothionein genes in hemocytes	(Corporeau and Auffret, 2003)
Ostrea edulis; C. gigas	*Bonamia ostreae*	Enzymatic activities – a commercial kit for detection of 19 enzymes	(Xue and Renault, 2000)
Ostrea edulis	*Bonamia ostreae*	Hemolymph function against *Bonamia ostreae*	(Cochennec *et al.*, 1992)
Pinctada fucata martensii	Post-operative care	Hemolymph hemagglutination activity	(Sano *et al.*, 2017)
Pinctada fucata martensii	Diseased oyster fluids	Hemocyte viability and survival of infected pearl oysters	(Morizane *et al.*, 2002)

Pinctada fucata martensii	Disease individuals	Hemocyte morphological changes	(Maeno *et al.*, 2001)
Pteria hirundo	*Vibrio* sp.	Hemocyte morphological and functional changes	(Vieira *et al.*, 2017)
Argopecten ventricosus	*Vibrio alginolyticus*	Antibacterial activity in the hemolymph	(Luna-Gonzalez *et al.*, 2007)
Chlamys farreri	Starvation	THC, ROS, acid phosphatase in hemolymph	(Xu *et al.*, 2008)
Chlamys farreri	T, salinity, air dry	Catecholamines in hemolymph	(Chen *et al.*, 2008)
Euvola (Pecten) ziczac	Pesticide	Cholinesterase activities of hemocytes	(Owen *et al.*, 2002)
Mytilus californianus	Diatom (Domoic Acid)	Changes in hemolymph pH, pCO_2, and pO_2	(Jones *et al.*, 1995)
Mytilus californianus	Tissue implantation	Hemocyte cell type, phagocytosis, and implant rejection	(Bayne *et al.*, 1979)
Mytilus coruscus	TiO_2	THC, viability, phagocytosis, lysosome, MN; MMP, ROS	(Wang *et al.*, 2019)
Mytilus edulis	Carbon nanofibers	Gene expression of hemocytes	(Barrick *et al.*, 2019)
Mytilus edulis	Heavy metals	Transportation of heavy metals in the circulatory system	(Devoid *et al.*, 2007)
Mytilus edulis	Air dry, hypoxia	Hemolymph acid-base status – pCO_2, ammonia, pO_2, and pH	(Booth *et al.*, 1984)
Mytilus edulis	Air dry	Changes of pH and pO_2 in hemolymph	(Jokumsen and Fyhn, 1982)
Mytilus edulis	Freshwater	Changes of the electrolyte contents	(Khlebovich *et al.*, 1981)
Mytilus galloprovincialis	Crude oil and dispersant	ROS, viability, phagocytosis, MXR, ACI	(Katsumiti *et al.*, 2019)
Mytilus galloprovincialis	Zinc pyrithione	THC	(Katalay *et al.*, 2019)
Mytilus galloprovincialis	Hypoxia	Cell type, ROS, viability	(Andreyeva *et al.*, 2019)
Mytilus galloprovincialis	Bacteria challenge	Bacterial adherence and association with hemocytes	(Zampini *et al.*, 2003)
Mytilus galloprovincialis	Estrogens	Vitellogenin levels in hemolymph	(Riffeser and Hock, 2002)
Mytilus galloprovincialis	BaP, 4NQO	DNA damage of hemocytes	(Bihari *et al.*, 1990)
Perna viridis	TiO_2, hypoxia	Cell type and number, THC, viability	(Wang *et al.*, 2014)
Perna viridis	Acute salinity	Hemocyte osmolality change	(McFarland *et al.*, 2013)
Perna viridis	Bacteria	Identification of a potent serine protease inhibitor	(Khan *et al.*, 2008)
Anadara senilis	Fluctuating Salinity	Osmotic pressure and ionic concentrations of hemolymph	(Djangmah *et al.*, 1979)
Laternula elliptica	Heat (10℃ for 48 hr)	Expression of HSP70 in thermally stressed hemocytes	(Park *et al.*, 2007)
Macrocallista nimbosa	Salinity	Viability, ROS, lysosome activity, phagocytosis	(Jauzein *et al.*, 2013)
Mercenaria mercenaria	QPX disease, T	ROS, phagocytosis, lysozyme activity	(Perrigault *et al.*, 2011)
Meretrix meretrix	$CdCl_2$, BaP	Identification of a small HSP gene and expression	(Li *et al.*, 2013)
Mya arenaria	Heavy metals	Phagocytosis, cell number, and viability	(Brousseau *et al.*, 1999)
Mya arenaria	Spatial and temporal change	Prevalence of leukemia in hemolymph	(Craig *et al.*, 1989)
Mya arenaria	*Bacillus megaterium*	Lipase activity in hemolymph and hemocytes	(Cheng and Yoshino, 1976)
Ruditapes decussatus	Dinoflagellate algae	Genotoxicity in gills and hemolymph	(Florez-Barros *et al.*, 2011)
Ruditapes philippinarum	Heat and cold shock	Identification of stress-immune response genes in hemocytes	(Menike *et al.*, 2014)
Ruditapes philippinarum	*Vibrio*	Cell count, viability, lysozyme activity, assay of bacterial infection	(Allam *et al.*, 2000)
Ruditapes philippinarum	*Vibrio*	Total protein and leucine aminopeptidase in hemocytes	(Oubella *et al.*, 1994)
Tapes philippinarum	Spatial and temporal change	Hematocrit, phagocytosis, lysozyme activity	(Matozzo *et al.*, 2003)
Tapes philippinarum	TBT	Superoxide dismutase, lysozyme activity	(Matozzo *et al.*, 2002)
Tridacna gigas	Repeated sampling	Total protein, ions composition, glucose, glycerol	(Rees *et al.*, 1993)

* T: temperature; TBT: tributyltin; BaP: benzo[a]pyrene; 4NQO: 4-nitroquinoline-N-oxide; TiO_2: titanium dioxide.
**THC: total hemocyte count; ROS: reactive oxygen species in cells; MN: mitochondrial number; MMP: mitochondrial membrane potential; MXR: multi-xenobiotic resistance; ACI: actin cytoskeleton disruption.

2. Total hemocyte number and proportions of different types

The total hemocyte number and relative proportions of different types were the most direct measurement of hemocyte immune responses. Although variations in hemocyte number may exist among individuals, any significant changes would be more likely linked to the metabolic condition changes. Counting of different types of hemocyte numbers could be accomplished by microscopic observation, but this method is time consuming with the process of making slides, staining, and counting. Flow cytometer is a fast and accurate method to count single-cell suspension such as sperm (Yang *et al.*, 2016) and can distinguish different types of hemocytes based on side scatter (SSC, measurement of cell granularity) and forward scatter (FSC, measurement of cell size) (Ashton-Alcox and Ford, 1998). Therefore, flow cytometry is becoming the mostly used approach for measuring the changes of hemocyte cell types.

3. Hemocyte viability

Hemocyte viability is a measure of the proportion of alive cells to evaluate the overall hemocyte health. Viability assays can be assessed based on cellular metabolism, enzyme activity, or cell membrane integrity. The widely used approach was double fluorescence staining with membrane permeable nuclear dyes, such as SYBR, and the membrane impermeable dyes, such as propidium iodide (PI), and detected by use of fluorescence microscopy or flow cytometry (Allam *et al.*, 2002). Alternatively, because cell membrane damage can cause release of cytosolic contents into the extracellular space including the enzyme lactate dehydrogenase (LDH), measurement of the extracellular LDH has also been used as an effective assay for hemocyte viability (Chu *et al.*, 2002).

4. Hemocyte apoptosis and cell cycle

Hemocyte apoptosis is a fundamental biological process in immune system for defensive functions (Sokolova, 2009). Hemocyte proliferation in cell number due to cell division in a sample can be used as an indicator to evaluate the cell health status. Therefore, apoptosis and cell cycle have been used as important assays for hemocytes in bivalves, such as in the eastern oyster against cadmium exposure (Sokolova *et al.*, 2004) and in the flat oyster *Ostrea edulis* against parasite *Bonamia ostreae* (Gervais *et al.*, 2018). Apoptosis and cell cycle assays could be performed by flow cytometry or genomic sequences.

5. Phagocytosis

Phagocytosis is the most fundamental role for hemocytes in bivalves to defense invasive pathogens, such as bacteria (Canesi *et al.*, 2002), and involves in collaboration of humoral defense factors such as agglutinins. Hemocytes can recognize, bind, and phagocytize the microbes, and the encapsulated microbes would be eventually degraded by cellular enzymes and oxidization to decrease the number of microbes.

Phagocytosis of hemocytes on foreign substances was firstly observed in the Pacific oyster (Feng, 1965), and has been reported to accomplish majorly by granulocyte hemocytes (Pipe, 1990), especially the eosinophil granulocytes (Hine, 1999; Anisimova, 2013; Pila *et al.*, 2016). The commonly used method to evaluate phagocytosis is to incubate fluorescence labelled beads (*e.g.* The Bangs Laboratories, Inc., https://www.bangslabs.com/) or actual microbes at certain temperature for a period of time, and the quantification of hemocytes with phagocytic beads can be performed by use of direct microscopic examination, fluorometric evaluation, or flow cytometry.

For bivalves, phagocytosis has been studied in many species (**Table 1**). The phagocytosis process involves in humoral defense factors such as agglutinins and lysosomal enzymes, and the surface-bound factors play a significant role in the bacteria-hemocyte interactions leading to the phagocytosis. Phagocytosis in bivalves can be affected by the environmental temperatures and other seasonal factors, but underlying factors influencing phagocytosis are still not completely understood (see a comprehensive review in Canesi *et al.*, 2002).

6. Reactive oxygen species (ROS) production

ROS are natural byproducts of the normal metabolism of oxygen in cell signaling and homeostasis. When exposure to environmental

stress, ROS production within cells would increase dramatically because of the damage to cell structures. After phagocytosis, the encapsulated microbes could be degraded by the oxidization process and cause ROS changes.

ROS production can be measured by use of a nonfluorescent analogue 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Eruslanov and Kusmartsev, 2010). After diffusing into the cells, DCFH-DA is hydrolyzed into 2',7'-dichlorofluorescein (DCFH) which would be trapped within the cells. The intracellular DCFH can be oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by ROS, and measurement of DCF fluorescence at 530 nm can be used to quantify the ROS production by use of a flow cytometer (Lambert *et al.*, 2003) or proteomic approach (Sheehan and McDonagh, 2008).

In molluscan bivalves, the production of ROS has been reported in hemocytes of many bivalve species, including oysters, mussels, scallops, and clams against environmental and biotic stresses (Donaghy *et al.*, 2012).

7. Lysosome enzyme activity

Lysosomes are membrane-bound vesicles containing digestive enzymes, such as glycosidases, proteases, and sulfatases, which can digest engulfed foreign microbes. In addition, lysosomes can destroy targeted organelles through autolysis, and be responsible for digesting protein from cell surface presented via endocytosis. Therefore, lysosome enzyme activity is a parameter to evaluate the status of hemocytes after phagocytosis.

In molluscan bivalves, the role of lysosomes following phagocytosis has been studied widely (Cheng, 1983). Lysosome enzyme activities was demonstrated in hemocytes of the north quahog after exposure and phagocytosis of single-cell algae (Moore and Gelder, 1985) and other stresses (**Table 1**). The measurement of lysosomal enzyme activities was usually performed by incubating with specific substrates and quantification of enzymatic products through comparing with negative controls without substrate (Moore and Gelder, 1985). Alternatively, probes linked to the factor controlling lysosomal homeostasis was identified and used as an effective and efficient tool for measuring lysosomal activity in mammalian cells (Ishii *et al.*, 2019), and may be applied for bivalve hemocytes.

8. Molecular pathways for hemocyte immunity

In recent years, molecular signal pathways for hemocyte immunity have been investigated in aquaculture bivalves. The molecular mechanisms for hemocyte immune recognition, signal transduction, and effector synthesis have been reviewed in two recent publications together with humoral immunity (Song *et al.*, 2010; Zhang *et al.*, 2019).

Application of Hemocyte Immunological Assays for Aquaculture

Environmental stresses, such as temperature, salinity, dissolved oxygen, pollutions, and red-tide algal toxins, are the challenges for molluscan aquaculture. To overcome these challenges, molluscan bivalves would close their shells as immediate responses and use their hemocyte immune system to respond (**Table 1**). However, with prolonged exposure to environmental stresses, molluscan bivalves could be subsequently susceptible to pathogens, increase disease outbreaks, and eventually suffer heavy mortality. Therefore, immunological responses of hemocytes in molluscan bivalves could show different levels and link to their considerable resilience to adverse environmental conditions. This suggests that, similar to the blood tests as diagnostic tool for health evaluation in human and livestock, hemocyte immunological assays in bivalves could be used as effective parameters to evaluate the impact of the environmental stresses, serve as measuring tools for genetic breeding, and provide diagnosis tools to guidance the operation management.

With the fast development of DNA sequencing technology, genomic tools such as immunological related genes, molecular pathways, and specific up- or down-regulation genes, have been investigated in responses to different environmental stresses. It is expected that combination of organism level, cellular, and molecular immunological assays could provide a full spectrum of immunological assays and serve as tools for improvement of molluscan aquaculture.

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Annotated Bibliography

(1) Anisimova A. A., 2013: Morphofunctional parameters of hemocytes in the assessment of the physiological status of Bivalves. *Russ. J. Mar. Biol.*, **39(6)**, 381-391.

This is a comprehensive review publication on shellfish hemocyte immunology. The basic morphology, cell type, cell function and dynamic changes of hemocytes in shellfish bivalves were summarized. The effects of temperature, season, salinity, annual cycle, food quality availability, toxin algae, bacteria and virus, pollutions, and heavy metals on hemocyte morphology, number and function were reviewed and documented.

(2) Donaghy L., Kim B. -K., Hong H. -K., Park H. -S., and Choi K. -S., 2009: Flow cytometry studies on the populations and immune parameters of the hemocytes of the Suminoe oyster, *Crassostrea ariakensis*. *Fish Shellfish Immunol.*, **27(2)**, 296-301.

This publication studied the immunological activities and morphology of hemocytes in the Suminoe oyster, *Crassostrea ariakensis* using flow cytometry and light microscopy. Three types of hemocyte types were identified, including hyalinocyte, granulocyte and blast-like cells. Cell count, survival, mortality, phagocytosis, and reactive oxygen species (ROS) production were evaluated using flow cytometer with different staining methods. It revealed that the granulocytes are most active in the cell phagocytosis and the hyalinocytes showed a certain level of the phagocytosis and oxidative activity, and the blast-like cells did not show any phagocytosis or oxidative activity.

(3) Vieira G. C., da Silva P. M., Barracco M. A., Hering A. F., de Albuquerque M. C. P., Coelho J. D. R., Schmidt E. C., Bouzon Z. L., Rosa R. D., and Perazzolo L. M., 2017: Morphological and functional characterization of the hemocytes from the pearl oyster *Pteria hirundo* and their immune responses

against *Vibrio* infections. *Fish Shellfish Immunol.*, **70**, 750-758.

This paper tested most hemocyte parameters including morphological characterization through light and electron microscopy and flow cytometry. Same as that in Sumino oysters, three types of the hemocytes were identified. Assays of phagocytosis and reactive oxygen species (ROS) production was performed by use of flow cytometer. Furthermore, hemocyte responses with exposure to a *Vibrio* pathogen was evaluated.

(4) Hégaret H., Wikfors G. H., and Soudant P., 2003: Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. *J. Exp. Mar. Biol. Ecol.*, **293(2)**, 249-265.

This paper studied the effects of temperature increase on hemocyte functions of eastern oysters, including aggregation, viability, phagocytosis, and ROS production. This is one of a series of publications on oyster hemocyte functions and detailed protocols were documented for readers to understand the analysis procedure. The results indicated that temperature increase (from 20 to 28 degree) caused no significant change in hemocyte aggregation, decreased the phagocytosis of all hemocyte types, induced significant hemocyte mortality in all hemocyte types, and increased, although not significantly, the ROS production.

(5) Jauzein, C., Donaghy L., and Volety A. K., 2013: Flow cytometric characterization of hemocytes of the sunray venus clam *Macrocallista nimbosa* and influence of salinity variation. *Fish Shellfish Immunol.*, **35(3)**, 716-724.

Salinity is one the most important factor potentially affecting shellfish physiology, especially in the inshore area with river flows. This paper estimated the hemocyte types and cellular parameters (oxidative activity, lysosomal content, phagocytosis capacity) in sunray venus clams, a potential aquaculture species in Florida. After exposure to salinities of 18, 21, 25, 30, 35 and 38 ppt for 7 days, hemocyte samples were collected and analyzed the parameters along with estimation of physiological status of clams, including mortality, valve closure, and filtration activity. It was interestingly found that hemocytes of sunray venus clam appeared as a unique population, both in terms of morphology and intracellular parameters. Clams after transferring to 18 and 21 ppt resulted in valve closure, mortality, and decreased filtration activity. Low salinities highly impacted hemocyte functions as follows: increased cell and lysosomal compartment volumes, decreased phagocytosis capacity, and increased oxidative stress and mortality.

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Preliminary feasibility assessment of purple sea urchin, *Strongylocentrotus purpuratus*, roe enhancement.

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Abstract: Sea urchin barrens and can stretch over 1000s of kilometers and last decades at a time. They are characterized by a predominance of urchins and coralline algae where kelp forests once existed. In contrast to barrens kelp forests provide habitat supporting thousands of vertebrates, invertebrates and plant species. Because kelp forests are keystone hosts their presence is vital to sustaining commercial and recreational industries including fishing and tourism. However, these kelp forests can collapse and shift to alternate stable states whereby urchin barrens persist. Over the last 4 decades, transitions between kelp beds and sea urchin barrens have been widely reported along temperate coastlines globally. During a kelp forest phase, urchin predation is the primary mechanism keeping sea urchin populations in check. However, due to various factors including climate change, predator densities can be reduced leading to shifts toward urchin barrens. Development of urchin fisheries has been implicated several times in recent history as a driver to return urchin barrens to kelp forests. However, this driver most recently has not worked in California and Oregon, USA where a large barren is persisting and expanding. Both states already had established urchin fisheries but it has been uneconomical for the fisheries to operate given urchins in the barrens had little gonad development or undesirable human consumption traits necessary for commercialization. Aquaculture in the US has potential to restore kelp forests by collaborating with fisheries to harvest wild urchins from barrens and fatten them in an aquaculture setting prior to sale. Although sea urchin roe enhancement is not a novel concept there are still technical limitations to the activity, primarily being the availability of macroalgae diets given seasonality and the propensity of urchin barrens to deplete kelp forests. Development of sustainable alternative diets for urchins is necessary for future commercial urchin aquaculture. In this student lead study, a preliminary replicated diet trial was performed for enhancing roe from purple sea urchin (*Strongylocentrotus purpuratus*) collected from California barrens using 4 diet treatments including giant kelp (*Macrocystis pyrifera*), ogo (*Gracilaria pacifica*), formulated commercial diet (Urchinomics), and an unfed control. During the 10 week study duration, gonadal somatic index (GSI) was measured in a subset of urchins (5 individuals) from each replicate tank every 2 weeks. Baseline GSI at the beginning of the trial was <0.5%. A GSI of 10% was reached most rapidly in the formulated diet treatment at 6 weeks, followed by ogo and kelp at 9 and 13 weeks respectively. This study was a preliminary examination of the feasibility of urchin ranching in California, showing biological potential for alternative diets to develop urchin gonads with a view to restore kelp forests and develop a nascent aquaculture industry in California.

Key words: urchin, barren, aquaculture, kelp, restoration

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Introduction

A sea urchin barren is a benthic habitat dominated by urchins, coralline algae, rocky substrate and largely devoid of macroalgae (Filbee-Dexter and Scheibling, 2014). Such barrens have been recorded throughout the world in coastal temperate marine waters and can range in size from 10s of m^2 to 1000s of km^2. Temporarily, urchin barrens are considered an alternative stable state of a marine ecosystem persisting in timescales from one year to several decades. The alternate stable state of the urchin barren is typically a kelp forest. There are marked differences in the biodiversity and general productivity of the urchin barrens and kelp forests. While urchin barrens are characterized by a predominance of urchins and coralline algae, kelp forests provide habitat supporting thousands of vertebrates, invertebrates and plant species. It is this keystone host role of kelp that make the presence of kelp forests the desired stable state for marine resource management as it supports commercial and recreational industries including fishing and tourism.

Over the last 4 decades, transitions between kelp beds and sea urchin barrens have been widely reported along temperate coastlines globally. During a kelp forest phase, urchin predation is the primary mechanism keeping sea urchin populations in check (Filbee-Dexter and Scheibling, 2014). However, due to various factors including both natural and anthropogenic, predator densities can be reduced leading to shifts toward urchin barrens. Unlike conventional animal population theory, once sea urchin grazing consumes the surrounding macroalgal biomass, the urchin population does not collapse or relocate, but rather urchin growth rate decreases and they enter a reproductive dormancy as a result of changing their feeding activity to less nutritious encrusting algae or microalgal biofilms (Lawrence, 1982). Hence, once formed, urchin barrens are a stable state and dominant feature of rocky reefs, surviving decades with individual urchins living up to 50 years on barrens (Ling and Johnson, 2009). Additionally, modelling research suggests that in order to return an urchin barren to a kelp forest state, urchin numbers must be reduced to abundances significantly lower than the urchin population before the phase switch to the barren state (Ling *et al.*, 2015). It is characteristics such as those described above that make urchin barrens persistent and long lived resulting in marked reduction in ecosystem services and negative economic effects on associated commercial and recreational industries (Rocha *et al.*, 2015).

Many sea urchin species around the world ordinarily represent valuable fisheries based on harvesting their roe for eventual consumption in gourmet restaurants. The roe is considered a delicacy in many cultures commanding market prices up to $248/kg in Japan (Stefánsson *et al.*, 2017). Economic analyses suggest that there is an unmet demand for good quality sea urchin roe. Considering the favorable economics of sea urchin fisheries, the occurrence of an overpopulation of sea urchins as seen in urchin barrens should represent an opportunity to increase fisheries landings and profits. However, as previously stated, once urchin barrens are established in an area, the macroalage that would ordinarily sustain their roe development is largely overgrazed resulting in the sea urchins entering a metabolic dormancy during which little if any roe is produced. This characteristic coupled with sea urchins' capacity to exist in low food conditions for extended periods make the conventional fishing of the sea urchins commercially unfeasible as a solution to remove and restore urchin barrens to kelp forests.

Aquaculture offers a potential economically viable solution to aid in the removal of sea urchins from barrens. While full life-cycle aquaculture exists for some sea urchin species it's development as an industry has been hindered by relatively slow growth (5 years to reach market size) impacting its profitability (James *et al.*, 2015; Unuma *et al.*, 2015; Williamson, 2015). In contrast, gonad enhancement of wild caught sea urchins can be achieved in relatively short time periods, 6 – 12 weeks (Heflin *et al.*, 2016). Sea Urchin gonad enhancement involves the capture of urchins with inferior roe qualities and subsequent culture of the animals to actively improve their roe qualities for market. Following capture of the wild sea urchins, gonad enhancement typically involves housing the animals in an aquaculture system and fed a diet designed to rapidly increase their gonad

size and improve their taste, texture and color commensurate with market expectations. With the recent increased prevalence and concern over sea urchin barrens, research efforts have increased to determine nutrition requirements and optimize culture systems for many species of sea urchins (Brown and Eddy, 2015).

Currently, a recently formed large sea urchin barren is affecting coastal kelp beds off California and Oregon, USA. It is estimated that purple sea urchin (*Strongylocentrotus purpuratus*) abundance in both states have increased 10,000% above baseline populations and have consumed over 90% of kelp coverage in northern California alone (Rogers-Bennett and Catton, 2019). This barren has attracted the attention of marine resource managers and agencies in both states looking to address its affects and restore kelp beds. The research detailed herein is a preliminary investigation into purple urchin (*S. purpuratus*) gonad enhancement using alternative feed stuffs as a potential tool to aid in the restoration of kelp beds in an economically viable manner.

Materials and Methods

The experiment was conducted at Moss Landing Marine Laboratories, California USA for a period of 10 weeks beginning in March, 2019 and concluding in May, 2019. 12 circular (1000 L) flow-through tanks (8 L/min) were used for the experiment with each one randomly assigned a diet treatment. The four diet treatments included an unfed control, a commercially formulated pellet diet (Urchinomics), giant kelp (*Macrocystis pyrifera*) and ogo (*Gracilaria pacifica*). There were three tank replicates of each treatment. The incoming seawater reflected nearshore conditions; with the temperature ranging from 10 - 17℃ and the salinity at 32 - 34 ppt. Tanks were aerated with air stones and dissolved oxygen maintained between 6 - 8 mg/L for the duration of the experiment. Each tank was initially stocked with 40 purple sea urchins, with a minimum test diameter of 39 mm. The urchins were collected from a nearby urchin barren in Monterey Bay by scuba divers, about a half a mile from shore. Before stocking the tanks, 30 urchins were randomly sampled to determine baseline values for test diameter and gonadosomatic index (GSI) (James *et al.*, 2017).

$$GSI = (\text{gonad wet weight}/\text{total weight}) \times 100$$

The urchins were fed to satiation with new feed added every 3 days providing ample feed within the tank to reduce searching behavior or periods where the urchins could not readily feed. Remaining uneaten feed after the 3 day period was removed from the tank and replaced with fresh feed. Mortality was negligible throughout the study and tanks were cleaned weekly to remove feces and biofouling on tank surfaces. Every 14 days, five urchins were randomly selected from each tank, dissected and GSI measured and recorded.

Changes in test diameter and GSI across time were calculated using a linear regression. In order to account for any tank effects, the average GSI from each replicate tank was used in the regression for each treatment. To predict the time it would take to raise an urchin to marketable roe content (GSI = 10%), the regression line equation was used. In order to find changes in GSI between the treatments over time while accounting for our hierarchical experimental design, a nested ANOVA using a linear mixed effects model was used. All assumptions about normality and homoscedasticity in the data and residuals were met. All statistics were conducted using R.

Results and Discussion

Expectantly, there were no changes in test diameter in any of the treatments across the course of the experiment (**Table 1**) due their reported relative slow growth. However, all of the treatments showed an increase in GSI across the course of the experiment (**Table 2**, **Fig. 1**). When accounting for the ranked experimental design, there were differences in GSI between the treatments themselves and differences across time. The effect of diet treatment on GSI also changed throughout the course of the experiment as a function of time (Nested ANOVA, Treatment: $F = 16.2$, $p = 0.0009$; Day: $F = 273.8$, $p < 0.0001$, Treatment: Day: $F = 15.9$, $p < 0.0001$). The urchins fed the formulated pellets showed the highest increases in GSI, followed

Table 1. *Change in test diameter.* The change in test diameter over time for each treatment was analyzed using linear regression

Treatment	Line Equation	df	F statistic	R^2	*p*-value
Control	y = -0.02x + 48.25	1, 14	0.296	0.005	0.589
Formulated	y = -0.04x + 48.36	1, 14	0.9	0.014	0.346
Kelp	y = 0.01x + 47.97	1, 14	0.08	0.001	0.781
Ogo	y = -0.04x + 48.74	1, 14	1.14	0.017	0.289

Table 2. *Change in GSI.* The change in GSI over time for each treatment was analyzed using linear regression

Treatment	Line Equation	df	F statistic	R^2	*p*-value
Control	y = 0.76x - 1.08	1, 14	261	0.952	5.51E-10
Formulated	y = 1.97x - 2.65	1, 14	272.63	0.954	4.20E-10
Kelp	y = 0.83x - 0.92	1, 14	46.5	0.781	1.23E-05
Ogo	y = 1.23x - 1.22	1, 14	158.77	0.924	1.16E-08

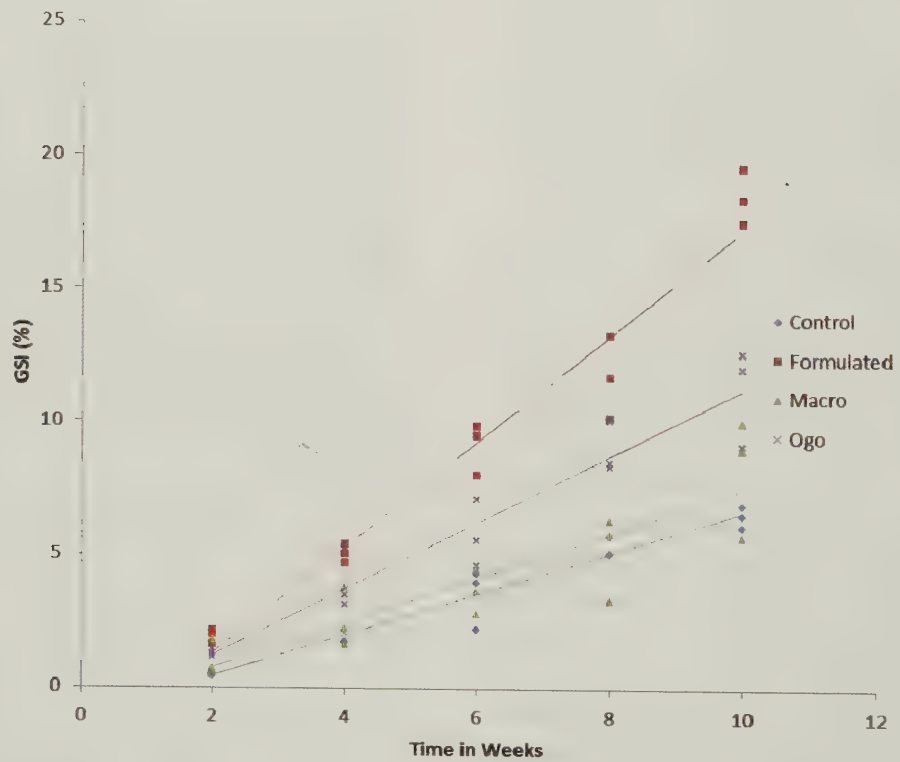

Fig. 1. Scatter plot of GSI values over time in weeks. Each data point represents a replicate tank average per treatment. Data points are distinguished to treatment level by a different color shapes/signs indicated in the figure key. Solid lines are linear regressions fitted to the respective treatment data points.

by those that were fed ogo. Interestingly, the urchins that were fed kelp showed similar growth to the control treatment wherein urchins were not provided any feed. Based on the GSI growth curves in each treatment, it would take 45 days (~6 weeks) to rear the urchins on formulated pellets to a marketable GSI of 10%. Similarly to reach 10% GSI it would take urchins 63 days (~9 weeks) fed an ogo diet, 91 days (~13 weeks) fed a kelp diet, and 101 days (~14 weeks) for urchins that are not

fed but consuming tank biofilm. Note that the final two predictions are outside the range of the linear regression for this study and thus likely unreliable.

Aquaculture roe enhancement of purple urchins is a potential tool for resource managers to restore kelp forests while also creating economic opportunities for both fisheries and aquaculture sectors. This activity also avoids wanton waste of a marine resource as is typical of urchin culling activities and the antithesis of resource management. Feed type is considered the most critical aspect of sea urchin roe enhancement as it heavily influences both the rate of roe production, texture, taste and color (Heflin *et al.*, 2016). All these characteristics are important to the economic feasibility of the activity. This preliminary study was conducted to examine gonad production rate for the biological feasibility of urchin roe enhancement in purple urchins collected from barrens off the California and Oregon coasts. While roe growth rate is only one aspect of roe enhancement the speed at which it develops will markedly affect profitability. The different feed types assessed for the study were selected to gauge relative growth rates on likely feed stuffs that could conceivably be used should the activity become established. They included an easily cultured seaweed (ogo) and a commercially formulated pellet diet (Urchinomics). The natural diet of kelp was also used as a type of positive control to compare their growth rates, although the authors note that due to the local presence of sea urchin barrens, kelp is not a viable feed stuff for roe enhancement. Similarly a negative control was also used, wherein no exogenous feed was supplied to the sea urchins.

Expectantly the pelleted diet treatment resulted in the most rapid roe production reaching a marketable GSI of 10% in just over 6 weeks. This is particularly noteworthy when considering the starting GSI of the sea urchins was >0.5%. Such a roe production rate is on par with some of the fastest roe enhancement rate reported elsewhere for other species (James, 2006). Also encouraging is that the ogo seaweed diet yielded relatively rapid roe production rates as well taking approximately 9 weeks to reach market size. The disparity between these two treatments is likely due to caloric density and digestibility being greater in the pellet diet compared to the seaweed diet (Cyrus *et al.*, 2013) but this was not assessed for the study. While the ogo treatment yielded relatively slower roe production rates compared to the pellet diet, the finding is still encouraging as the duration is within a time period reported as acceptable for other roe enhancement research. Furthermore, the feeding of a live seaweed capable of vegetative/fragmentation growth like ogo, offers other benefits including excellent feed stability and longevity, ability to maintain continuous culture onsite and potentially extract dissolved nutrients from the water column as a result of sea urchin culture. These characteristics and general simplicity of the diet could complement the artisanal nature typical of commercial urchin fisheries, affording fishers an opportunity and low entry cost to enhance their wild caught sea urchins. In contrast the kelp positive control treatment and negative control treatment curiously yielded much slower but similar roe production rates. A potential explanation for the unfed control treatment displaying roe growth rates is a verification of sea urchins hardiness. The negative control tanks experienced observable biofouling between the weekly cleanings and thus it is theorized that the biofilm on the tank surfaces provided enough nutrition to support modest roe production. As all tanks in the experiment experienced the same biofouling activity it is unlikely to have confounded the results of the experiment overall.

In conclusion this preliminary research has indicated that rapid roe enhancement of purple urchin derived from urchin barrens in California, USA is feasible using both a formulated pellet feed and a live red seaweed species. While it was not in the scope of this preliminary experiment, future research for roe enhancement of purple urchins should include a more thorough assessment of nutrition requirements including proximate analysis of feed types, measurements of digestibility and intake rate as well as quantitatively assessing the resulting roe for important market characteristics including color, texture and taste. Based on the outcome of these and other biological considerations of purple urchin (*S. purpuratus*) roe enhancement, an economic feasibility is also required to determine the commercial potenital of this aquaculture activity for

transforming urchin barrens back to kelp forests.

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This paper provides an excellent description of sea urchin barrens as to what they are comprised of, and their extent both temporally and spatially across the world. The authors examine and list the drivers of phase shifts between barrens and kelp forests.

They describe different thresholds for forward (to barrens) and reverse (to kelp beds) shifts, in accordance with alternative stable-state dynamics. They surmise that accelerating climate change and increasing anthropogenic impacts play important roles in altering alternative stable-state dynamics and triggering phase shifts.

(2) Heflin L. E., Makowsky R., Taylor J. C., Williams M. B., Lawrence A. L., and Watts S. A., 2016: Production and economic optimization of dietary protein and carbohydrate in the culture of Juvenile Sea Urchin *Lytechinus variegatus*. *Aquaculture* **463**, 51–60. (doi. org/10.1016/j.aquaculture.2016.05.023)

This manuscript is a through collection of urchin feeding experiments to understand the nutrient requirements of urchins for aquaculture. The paper creates predictive models of growth, production and efficiency outcomes and generates economic analysis models in relation to these dietary outcomes for urchins held in culture. The models compare dietary requirements and growth outcomes in relation to economic costs and provide insight for future commercialization of sea urchin aquaculture

(3) Unuma T., Sakai Y., Agatsuma Y., and Kayaba T., 2015: Sea Urchin Aquaculture in Japan, in "Echinoderm Aquaculture" (ed. by Brown N. P., and Eddy S. D.), John Wiley & Sons, Inc, Hoboken, New Jersey, pp.75–126. *Wiley Online Library* (doi. org/10.1002/9781119005810.ch5)

This is the most recent review of urchin aquaculture in Japan. Japan is the foremost consumer of urchins and sigificnfact producers of urchins both from ranching and closed life cycle aquaculture. The chapter details the history of urchin fisheries in Japan and the rise of urchin barrens and urchin aquaculture. The review discusses the diver mediated destruction of urchins to bring back kelp beds as well as reseeding efforts to restore overfished urchin grounds. The review also discusses the development of full life-cycle aquaculture to meet both reseeding and commercial production requirements. Also the movement of urchins from barrens to other kelp grounds and aquaculture facilities for commercial fattening are detailed.

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Bull. Jap. Fish. Res. Edu. Agen. No. 50, 55－66, 2021

Potential impacts and management of ocean acidification on Japanese marine fisheries and aquaculture.

Haruko KURIHARA*

Abstract: Marine fisheries have great importance economically and for food production, particularly in Asian countries including Japan. Although the total value of fisheries production has decreased in Japan since 1982, it still has enormous commercial value accounting for about US$157 billion (JPY15,700 billion). Additionally, the world demand for seafood is expected to further increase with the growth of human population and income in developing countries. Ocean acidification (OA), which is caused by the increase of atmospheric CO_2, is now an increasing cause for concern as a major threat to marine fisheries together with global warming. Because OA causes a decrease in calcium carbonate saturation, most marine calcifiers, which include a number of commercially important shellfish such as mollusks and crustaceans, are expected to be particularly affected by OA. In this study, an overview is provided of the scientific knowledge of OA with regard to commercially important organisms and the potential impacts on marine fisheries and aquaculture in Japan. Potential management and adaptive strategies to mitigate impacts of OA on marine fisheries in Japan are also discussed.

Key words: ocean acidification, fisheries, Japan, aquaculture, calcifiers

Introduction

Marine fisheries have great importance economically and with food production, particularly in Asian countries including Japan. Seafood consumption has steadily risen since the 1960s (FAO, 2009) and reached around 80 million tonnes recently (Watson and Pauly, 2001). The demand for seafood is expected to further increase with the growth of the human population and income in developing countries. Continuously increasing pressure on supply of marine food sources is a principal threat to future fisheries. In addition to fishing activities, impacts of climate change are emerging as another serious challenge for the marine fisheries industry (Brander, 2007). A number of studies have shown that global environmental change can severely affect the entire marine ecosystem and directly affect economically important fish and shellfish species (Allison *et al.*, 2009, Cheung *et al.*, 2010). Rising temperature due to global warming is now causing changes in species distribution and productivity of the ocean (Cheung *et al.*, 2013). In addition, "ocean acidification (OA)" is being highlighted as a threat to marine fisheries and aquaculture (Doney *et al.*, 2009). In this paper, an overview is provided regarding the potential impacts of ocean acidification on marine fisheries and aquaculture in Japan, which is one of the world's largest fishery industries. Additionally, potential management and mitigation solutions are discussed.

Background of Ocean Acidification

Since the Industrial Revolution, atmospheric carbon dioxide (CO_2) concentration has steadily increased from an average of 280 μatm to the present 400 μatm value (IPCC, 2014). Increased atmospheric CO_2 is quickly absorbed into the seawater and dissolved into bicarbonate ion (HCO_3^-)

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or carbonate ion (CO_3^{2-}) and hydrogen ions (H^+). Therefore, seawater becomes more acidic with an increase in atmospheric CO_2 (Caldeira and Wickett, 2003). The present seawater average pH (8.1) has already decreased by 0.1 unit since the Industrial Revolution, and IPCC scenarios (RCP2.6–8.5) expect the pH to decrease by another 0.1–0.4 units by the end of this century (IPCC, 2014). According to seawater carbonate chemistry, as the seawater pH decreases, CO_3^{2-} reacts with H^+ forming HCO_3^- and hence the CO_3^{2-} concentration ($[CO_3^{2-}]$) decreases. Because seawater calcium carbonate saturation (Ω) is defined by the amount of calcium ion concentration ($[Ca^{2+}]$) and $[CO_3^{2-}]$, Ω will also decrease with OA.

$$\Omega = [Ca^{2+}]\,[CO_3^{2-}] / K_{sp}^*$$

(K_{sp}^* = calcium carbonate solubility product)

The decline of Ω is suspected to affect marine calcifiers including mollusks, echinoderms and crustaceans. For the formation of calcium carbonate ($CaCO_3$) shells and skeletons, marine calcifiers actively excrete H^+ by ATPase pumps to increase the Ω at the compartment they extract $CaCO_3$ (Allemand *et al.*, 2011). Therefore, OA is expected to affect the $CaCO_3$ production of marine calcifiers because they will need more energy to compensate for acidosis at those compartments (Al-Horani *et al.*, 2003; Cohen and Holocomb, 2009). In addition to affecting calcification processes, many other biological processes, such as metabolism and protein synthesis, are also suspected to be affected by OA because acidosis can cause the decline of intracellular pH unless the organism has the ability to compensate their internal acid-base balance (Pörtner and Farell, 2008). Therefore, organisms that have less compensation capacity, such as marine invertebrates and those in early developmental stages, are expected to be particularly affected by OA.

Potential Impacts of Ocean Acidification on Marine Fisheries in Japan

The Japanese fisheries catch amount steadily increased since 1960 and reached a peak (12.7 million t) around 1982, however, the fisheries catch amount has since decreased to present levels of around 4 million t (MAFF, 2019) (**Fig. 1**). Nevertheless, the present value of Japanese fisheries is still of major commercial importance with a total value of about US$157 billion (JPY15,700 billion as of 2018). Japanese marine fisheries are mainly classified into 3 categories: marine fisheries, marine aquaculture and island fisheries. Marine fisheries are further divided into 3 categories according to the location they are conducted: distant marine fisheries operated at high seas and foreign countries EEZ, offshore marine fisheries operated within Japan's and neighboring countries EEZ, and coastal fisheries operated in Japanese coastal waters. The clear trends in Japan's fisheries are that the catch amount in the distant and offshore marine fisheries have decreased from 2.3 and 6.7 million t in 1986 to 0.3 and 2.0 million t in 2018, respectively (**Fig. 2**). During this same period, the amount of aquaculture has remained stable and the relative percentage value of aquaculture to the total fisheries value has steadily increased since 1960 from 10% to about 38% with a total value of about US$60 billion (MAFF, 2019) (**Fig. 2**). Therefore, the Japanese fisheries is relying more and more on coastal fisheries and aquaculture than ever before.

Though finfish are still the main target of

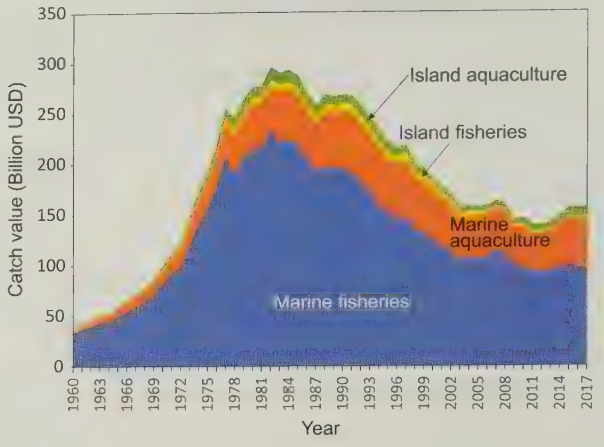

Fig. 1. The transition of marine fisheries, marine aquaculture, inland fisheries and inland aquaculture total catch values in Japan since 1960. The fisheries catch value steeply increased since 1960 and peaked in 1982. Since then the catch value continuously decreased to the present value of about US$157 billion (JPY15,700 billion).

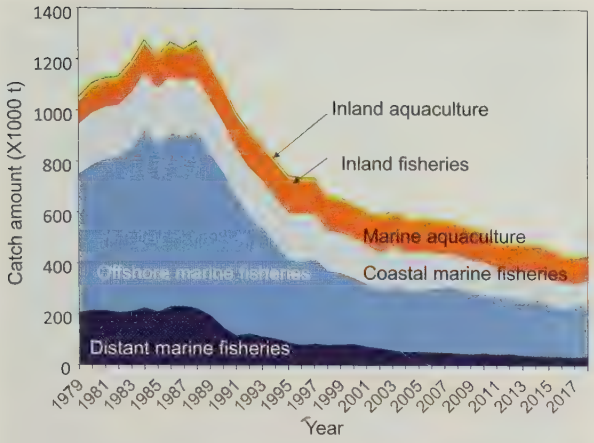

Fig. 2. The transition of catch amount of distant marine fisheries, offshore marine fisheries, coastal marine fisheries, marine aquaculture, inland fisheries and inland aquaculture since 1979. The catch amount of distant and offshore marine fisheries steeply decreased since 1984.

Japanese marine fisheries, the proportion of other organisms has increased primarily after the collapse that occurred after 1982, and now other organisms account for about 30% of the total catch value. These include mollusks such as scallops and oysters, crustaceans including shrimps, crabs and krill, sea urchins, cephalopods and seaweeds (**Table 1**). In particular, many shellfish are now important aquaculture species and mollusks account for 20% of the total catch value (about US$9.5 billion). An important concern is that many of these organisms are highly susceptible to OA conditions.

1. Impacts on mollusk fisheries

Mollusks are the second largest group of commercial fisheries organisms next to finfish, and the catch value accounts for more than US$17 billion (MAFF, 2019). The most economically important mollusks in Japanese fisheries include scallops, abalones, oysters and clams. Scallops in Hokkaido and oysters in Miyagi and Seto Inland Sea have the greatest market values for mollusks in Japan. Oysters are mainly cultivated through aquaculture, while scallops are both wild caught in coastal waters and farmed.

The effect of OA on mollusks was first demonstrated in the mineralization of Pacific oyster (*Crassostrea gigas*) larval shells (Kurihara *et al.*, 2007). Veliger larvae of the oysters were found to become smaller in size, and shell mineralization was completely inhibited in 45% of the larvae reared under high CO_2/low pH conditions (Kurihara *et al.*, 2007). Recently, a number of studies demonstrated similar effects of OA on the larval stage of several different mollusk species including oysters (*C. virginica, Saccostrea glomerata*), scallops (*Argopecten irradians*), hard clams (*Mercenaria mercenaria*), mussels (*M. galloprovincialis, M. edullis*) and abalone (*Haliotis coccoradiata*) (Kurihara *et al.*, 2008a; Tamalge and Gobler, 2009; Parker *et al.*, 2009; Gazeau *et al.*, 2010; Byrne *et al.*, 2010). Additionally, fertilization rate of scallop eggs (*Mimachlamys asperrima*) was found to decline with high CO_2/low pH but not for oysters (Scanes *et al.*, 2014; Havenhand *et al.*, 2008). Because early life stage survival is the bottleneck to the recruitment of most marine invertebrates, these studies indicated that OA could strongly impact the population size of many commercially important species. OA was also reported to affect the calcification rate of adult oysters (*C. gigas*), mussels (*M. edulis*), and adult scallops (*Argopecten irradians*) (Gazeau *et al.*, 2007; Ries *et al.*, 2009). The immune system of adult *C. gigas* was found to be significantly affected by high CO_2/low pH, suggesting that OA can increase their disease susceptibility (Wang *et al.*, 2016). Synergistic effects of high CO_2/low pH and high temperature were also found in both oysters and scallops. These studies indicated that global warming and ocean acidification could intensify their negative effects on these organisms (Parker *et al.*, 2009;

Table 1. Japan catch value of marine fisheries and aquaculture in 2017. Values are shown in billion U.S.D

	Marine fisheries	Marine aquaculture
Fish	66.7	25.2
Mollusks	9.0	9.5
Crustacean	6.1	0.7
Sea urchin	1.2	–
Cephalopod	8.9	–
Seaweed	2.0	14.1
Others	1.9	0.1
Total	96.0	49.7

Schalkhausser *et al.*, 2014). From these studies, most mollusk fisheries including oysters and scallops have been suggested to be at high risk under OA conditions. This prediction was confirmed in 2009, when U.S Pacific Northwest Pacific oyster (*C. gigas*) hatcheries experienced a substantial production failure of *C. gigas* larvae production (Pacific Coast Shellfish Growers Association, 2010). This loss was interpreted to be related to the fact that the year's intense upwelling brought high CO_2 deep seawater up to the surface which acidified the seawater used at these hatcheries. This has highlighted the potential extensive effects of OA on oyster fisheries that may occur all over the world, including Japan, and the importance of considering adaptive strategies to mitigate OA impacts.

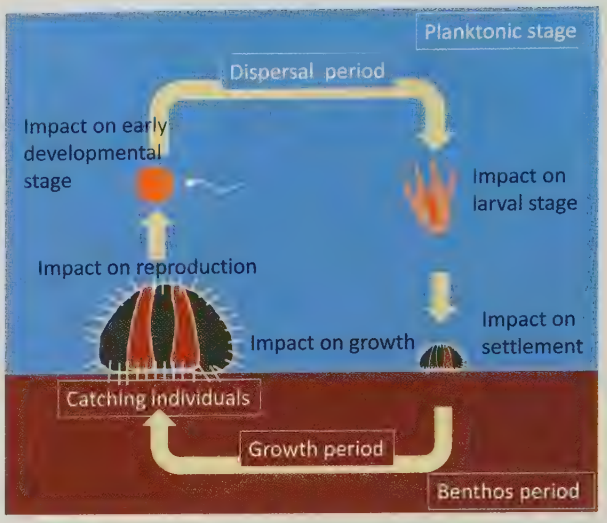

Fig. 3. Ocean acidification impacts on the whole life cycle of a sea urchin. OA can affect several different life cycle stages and different biological activities.

2. Impacts on sea urchin fisheries

Sea urchins are also commercially important organisms in Japan, where the gonads are eaten as "sushi" topping and have a catch value of about US$1.2 billion. In Japan, they are mainly caught around the northern island, Hokkaido, and the main commercial sea urchin species are *Strongylocentrotus intermedius* and *S. nudus*, though other sea urchins such as *Pseudocentrotus depressus, Anthocidaris crassipina* and *Tripneustes gratilla* are also caught.

Effects of OA on sea urchins are also well studied and represent some of the first studies that demonstrated the effects on early life stages (Kurihara and Shirayama, 2004; Kurihara *et al.*, 2004; Kurihara 2008). When eggs of the sea urchin *Hemicentrotus pulcherrimus* and *Echinometra mathaei* were reared under high CO_2/low pH seawater, it was found that the fertilization rate and developmental speed of the embryos decreased (Kurihara and Shirayama, 2004; Kurihara *et al.*, 2004). Additionally, the larval skeleton formation and larval size of sea urchins were found to be negatively affected by OA, which potentially results in the decrease of their population size (Kurihara and Shirayama, 2004; Kurihara *et al.*, 2004). Larval physiology was also found to be affected by OA and similar results were found in a number of other sea urchin species, including *Strongylocentrotus drobachiensis, S pupuratus, S. franciscanus, S. intermedius, Heriocidaris erythrogramma, Paracentrotus lividus, Pseudechinus huttoni, Evechinus chloroticus, Sterechinus neumayeri, Arbacia dufresnei, Centrophanus rodgersii, T. gratilla* (O'Donnell *et al.*, 2010; Brennand *et al.*, 2010; Clark *et al.*, 2009; Stumpp *et al.*, 2011; Martin *et al.*, 2011; Foo *et al.*, 2012; Byrne *et al.*, 2013; Zhan *et al.*, 2016). Growth and survival of sea urchins were reported to decline with OA in not only planktonic larval stages but also during juvenile stages (Shirayama and Thornton, 2005). Gonad development and physiology of adult sea urchin, *H. pulcherrimus*, were also found to decrease when cultured for 9 months under high CO_2/low pH seawater (Kurihara *et al.*, 2013). Similarly, reduced growth and poor gonad production were observed in *T. gratilla* (Mos *et al.*, 2016), suggesting that OA can directly reduce both the product value and the productivity of sea urchins. Test thickness and strength were also found to be affected by OA, suggesting less resistance of sea urchin to predation (Byrne *et al.*, 2014). The immune system was also found to be affected by OA, indicating their higher susceptibility to pathogens (Leite Figueiredo *et al.*, 2016). These studies also suggested that OA can affect several different life stages and different biological activities, impacting their population size (**Fig. 3**).

3. Impacts on crustacean fisheries

There are several crustaceans that are consumed

as a food source in Japan, and the most commercially important species include Japanese spiny lobsters (US$64 million), prawns (US$18 million), snow crabs (US$192 million), Japanese blue crabs (US$23 million) and krill (US$13 million). The impacts of OA on crustaceans seem to be highly variable among species, where some species show strong tolerance to high CO_2/low pH, while some other species show high sensitivity (Whiteley, 2011; Kroeker *et al.*, 2010). In terms of commercially important species, Ries *et al.* (2009) reported that the calcification of the juvenile American lobster (*Homarus americanus*) was not affected by high CO_2/low pH conditions. However, growth rate and mineralization of carapace of larvae and postlarvae of American lobster and European lobster (*H. gammarus*) have been found to be affected by OA (Arnold *et al.*, 2009; Keppel *et al.*, 2012). Additionally, the immune response of the Norway lobster (*Nephrops norvegicus*) has been found to be suppressed by high CO_2/low pH, suggesting a potential increase of disease susceptibility of lobsters due to OA conditions (Hernroth *et al.*, 2012). Although there is still no study evaluating the effects on Japanese spiny lobster (*Panulirus japonicus*), which is one the most commercially important crustaceans in Japan, these studies suggest that OA can potentially impact the lobster fisheries in Japan. Low seawater pH significantly decreases embryonic development and hatching success on the Florida stone crab (*Menippe mercenaria*), while the effect on hatching was highly variable, suggesting that there are some differences in tolerance capacity among individuals (Gravinese, 2018). Negative impacts on growth and survival rate of juvenile Red King Crab (*Paralithodes camtschaticus*) and snow crab (*Chionoecetes bairdi*) exposed at high CO_2/low pH condition were also observed (Long *et al.*, 2013). Punt *et al.* (2016) conducted a model analysis evaluating the potential effects of expected future OA conditions on the Tanner crab fishery and estimated that catch and profits would decrease by 50% over the next 20 years if survival of the crabs is affected by high CO_2 conditions.

Ocean acidification has also been observed to affect several prawn species, including *Palaemon pacificus*, *Penaeus serratus*, *P. elegans*, *P. monodon*, *P. occidentalis*, and *Melicertus plebejus* (Wickins, 1984; Kurihara *et al.*, 2008b; Kikkawa *et al.*, 2008; Ries *et al.*, 2009; Dissanayake *et al.*, 2010). Only short-term impacts of extremely high CO_2 conditions (3–15%) were studied on juvenile *Marsupenaeus japonicus*, results of which showed high tolerance to CO_2 (Kikkawa *et al.*, 2008); however, long-term effects should also be studied because the effect of 1,000 μatm CO_2 on *P. pacificus* only became apparent after 30 weeks of exposure. Furthermore, not only are commercially important crustaceans as a human food source affected by OA, but also trophically important prey species such as copepods and Antarctic krill, suggesting critical impacts of OA on entire marine ecosystems (Kurihara *et al.*, 2004; Kawaguchi *et al.*, 2010; Cripps *et al.*, 2015)

4. Impacts on cephalopod fisheries

Only a few studies have evaluated the effects of OA on cephalopods, including octopus and squid. While reduced metabolism was reported for jumbo squid *Dosdicus gigas* (Rosa and Seibel, 2008), neither calcification nor growth of the cephalopod *Sepia officinalis* was affected by high CO_2/low pH seawater and they showed efficient ability to regulate acid-base status (Gutowaska *et al.*, 2009). However, high CO_2/low pH was observed to depress energy expenditure rate of *S. officinalis* embryos (Rosa *et al.*, 2013). Additionally, increased time to hatching and effects on statoliths were found on the commercially important squid *Doryteuthis pealeii*, reared under high CO_2/low pH conditions, suggesting impacts of OA on their behavior and survival (Kaplan *et al.*, 2013). A more recent study evaluating the swimming behavior of paralarval *D. pealeii* by three-dimensional video system did not show clear effects of high CO_2 conditions on the swimming behavior (Zakroff *et al.*, 2018).

5. Impacts on seaweed fisheries

Seaweed aquaculture has great commercial importance in Japan and the farmgate value of red algae, *Pyropia spp.*, collectively called "Nori" in Japanese language, accounts for about US$11.6 billion. *Undaria pinnafida* called "Wakame" and Japanese kelp such as *Saccharina japonica* called "Konbu" are also commercially important seaweeds

harvested in Japan. In contrast with most marine calcifiers, seaweeds are expected to be positively affected by OA, because increases in seawater pCO_2 potentially increases their photosynthetic rate and productivity. However, the majority of seaweeds are known to use both CO_2 and HCO_3^- for carbon fixation and have evolved a carbon concentrating mechanism (CCM) and hence may not be positively affected by the increase of seawater pCO_2 (Koch *et al.*, 2013). For example, although many seagrass species such as *Zostera marina* show increased productivity under OA conditions (Palacios and Zimmerman, 2007), the photosynthetic rate and productivity of the giant kelp *Macrosystis pyrefera* did not change with seawater pCO_2 (Fernandez *et al.*, 2015). Therefore, seaweed fisheries are suggested not to be negatively or positively affected by OA, though increased temperatures would affect these species. Meanwhile, although seaweed may have less capacity of sequestering carbon compared to the seagrass, seaweed farms can potentially work as pCO_2 offset by being harvested as human resources (Froehlich *et al.*, 2019). Particularly, taking into account the market size of seaweed farming in Japan and other Asian countries, seaweed fisheries may potentially work as one of the strategies for sustainable ocean use.

6. Impacts on fish fisheries

Marine fishes have been thought to be tolerant to OA because of their high capability to regulate acid-base balance and the internal pH at high pCO_2 seawater (Kroeker *et al.*, 2013; Wittmann and Pörtner, 2013). Branchial cells are known to actively excrete ions through the Na^+/K^+-ATPase channels using energy to regulate the internal pH (Pörtner and Peck, 2010). However, more recent studies demonstrate that fish in early life stages with less developed acid-base regulation capabilities can be highly vulnerable to OA. For example, Atlantic herring (*Clupea harengus* L.) embryos show a decline in protein biosynthesis when reared under a high CO_2/low pH conditions (Franke and Clemmesen, 2011). Additionally, high CO_2/low pH conditions were reported to cause tissue damage on various organs including the liver, pancreas and kidneys of Atlantic cod (*Gadus morhua*) larvae (Frommel *et al.*, 2011). Survival rate of *Menidia beryllina* in early life stages was demonstrated to decrease by 73% even at 780 μatm CO_2 seawater (Baumann *et al.*, 2012). Meta-analysis using data obtained from different fishes indicated that high sensitivity on early life stages were broadly observed particularly on pelagic and euryhaline fishes (Cattano *et al.*, 2018). The size of otoliths made of $CaCO_3$ was found to increase in several fishes, though it is still not clear if this change affects sound detection (Munday *et al.*, 2011; Heuer and Grosell, 2014). Furthermore, sensory lateralization, learning ability, predator avoidance behavior and spatial orientation have been found to be disrupted under high CO_2/low pH conditions particularly in tropical fishes (Munday *et al.*, 2009, 2014; Dixson *et al.*, 2015). Moreover, even though most adult fishes have the capability to compensate the acid-base balance when reared under high CO_2 conditions, long-term exposure to high CO_2/low pH conditions are suggested to affect fish energy budgets because the regulation of pH is energetically costly (Heuer and Grosell, 2016). Furthermore, although low growth rates of fish larvae have been reported (Frommel *et al.*, 2016), no clear effects have been found on swimming speed or standard metabolic rate of most adult fishes (Melzner *et al.*, 2009; Lefevre, 2016; Esbaugh, 2018).

Potential Management and Solutions

With the increase of scientific knowledge regarding the effects of high CO_2/low pH seawater on many marine organisms, it has become evident that Japanese fisheries are likely greatly impacted by OA. However, much research is still needed to better understand OA in the context of Japanese fisheries including topics such as the present OA conditions in Japanese coastal waters, presence of hotspots, potential populations highly vulnerable or more tolerant to OA, the effects of multiple stress exposure on organisms, and many others.

Because coastal waters are strongly affected by the land through receiving freshwater and organic inputs, seawater carbonate chemistry can be highly spatially and temporally variable compared to open water. Therefore, different locations can have different seawater carbonate chemistry. There is a

fundamental need to know the carbonate chemistry of each location of interest to predict and manage the potential impacts of OA on the local marine fisheries. Nevertheless, locations that have higher seawater residence times, such as inner bay areas and locations that show higher organic carbon decomposition, are suggested to have higher risk to OA. This is because remineralization of organic matter by bacteria can decrease seawater oxygen concentration and increase CO_2 concentration causing local hypoxia and hypercapnia concurrently. For example, pH and Ω in Tokyo Bay was found to be lower in bottom water than in surface water because of remineralization of organic carbon (Yamamoto-Kawai *et al.*, 2015). Additionally, low pH and low oxygen was found to occur in bottom water in Shizugawa Bay during the summer (Kurihara *et al.*, unpubl.). This suggests that organisms living in these locations can be exposed to increased OA and low oxygen simultaneously and may be synergistically affected by these stressors. Additionally, such conditions put benthic organisms at particularly high risk during summer seasons. These data can be highly informative for aquaculture site selection and early detection of OA risks to the marine fisheries. Additionally, reduction of eutrophication can be effective in reducing the risk of both OA and hypoxia.

Along with environmental factors, further biological data and assessment models for evaluating the effects of OA on commercially important marine fisheries are essential for better management. Recent studies have focused on using socioeconomic models to predict the population of shellfishes, such as scallops and red king crab, under different CO_2 emission scenarios (Cooley *et al.*, 2015; Punt *et al.*, 2014). These studies can provide important information for decision makers and fisheries managers for better fisheries management in dealing with climate change. There is still a lack of biological data and very little information on the effects of OA on the entire life cycle of a species. Furthermore, there is little data regarding long-term effects on reproduction of many commercially important species, which could be the focus of future studies. Additionally, there are still several commercially important species where there is no information known regarding OA effects, such as the sea urchin *S. nudus*, the Japanese spiny lobster (*P. japonicus*), seaweeds and most marine fishes.

For aquaculture species, existing scientific data can be used for better management and solutions to mitigate OA impacts. For example, controlling seawater pH in hatchery tanks or within fish cages is possible by adding alkaline solutions or bubbling controlled gas. Additionally, avoidance of crowded fish cages or tanks, particularly during the summer season, can decrease the risk of hypercapnic conditions. Because many studies revealed that the early life stage of most marine organisms are particularly vulnerable to OA (Kurihara, 2008), management efforts can be focused on those stages. Several studies also demonstrated that when adults are reared under high CO_2, the next generation can show higher tolerance to CO_2. For example, larvae of Sydney rock oyster *Saccostrea glomerate* spawned by adults cultured under high CO_2 conditions, show higher growth rate compared to the larvae spawned by individuals cultured under control conditions (Parker *et al.*, 2012). Additionally, as already mentioned, many studies also demonstrated that tolerance capacity to OA can vary among individuals. Therefore, selective breeding of OA resistant strains could be one potential solution for aquaculture.

Finally, even though many scientific studies are now available on the effects of OA, general concern for OA impacts on Japanese fisheries is still limited. Further scientific efforts addressing the issues caused by OA for use by decision makers and fisheries and environment management sectors in Japan is essential to develop adaptive strategies and to be prepared for environmental changes in the near future.

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Sustain seafood resources in the U.S. affiliated Pacific islands- status and strategies

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Abstract: Seafood plays important crucial socio-economic roles in daily life of the U.S.-affiliated Pacific Islands (USAPI). USAPI includes American Samoa, the Republic of the Marshall Islands (RMI), the Federated States of Micronesia (FSM), the Commonwealth of the Northern Mariana Islands (CNMI), Guam, and the Republic of Palau (Palau). With less than 2,558 km^2 in total land mass but extended Exclusive Economic Zone (EEZ), the primary source of dietary protein for Pacific islanders has to come from the Ocean. The per capita seafood consumption exceeded global average. In 2015, per capita seafood consumption in Oceania was 25.0 kg/year vs 15.5 kg/year worldwide (FAO, 2018).

Seafood comes from capture fisheries and aquaculture but capture fisheries are the main seafood source for the USAPI. Increasing threats from climate change and overfishing have diminished the sustainability of yield from capture fisheries. Although there were optimistic reports on status of tuna stocks (key species in capture fisheries) in the Pacific, foreign fishing companies have exported majority of their catch to consumers outside the islands. Islanders have relied on subsistence catch to meet their demand. As the harvest from nearshore fishery declined, they have to consider secure their seafood supplies from other means such as aquaculture.

USAPI can take the advantages of their superior natural resources, such as pristine water, year-round warm weather, and isolated condition for disease prevention to sustain their seafood sources via fish farming. However, they have to overcome constraints to aquaculture development such as small land area, natural hazards for some islands, insufficient knowledge base, shortage of available capital, distant markets, and poor transportation systems. Except natural hazards for some islands, the other constraints are solvable. A good strategic development plan is essential to reveal aquaculture potential in the region.

This report discuss the potential threat of climate change to fishery, review the current status and challenges of aquaculture, and finally present some suggestions on future development.

Key words: seafood, aquaculture, sustainability, climate change, US affiliated Pacific Islands, food security

Introduction

Seafood comes from both capture fisheries and aquaculture. In 2016, capture fisheries and aquaculture contributed equally to the total global seafood production. For the U.S.-affiliated Pacific Islands (USAPI), capture fisheries remains the main source of seafood supplies. Seafood plays a key important role in supporting livelihood of the USAPI. USAPI include American Samoa, the Republic of Marshall Islands (RMI), the Federated States of Micronesia (FSM), the Commonwealth of the Northern Mariana Islands (CNMI), Guam, and the Republic of Palau (Palau). This region, composed of thousands of tiny islands spread between the latitudes of 15° N to 14° S and the longitudes of 134° E to 170° W, has less than 2,558 km^2 in total landmass. With their Exclusive Economic Zone

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(EEZ), it extends across an area as large as the continental United States. This extended EEZ is their primary nature resource for living and economic development. Their ancestors have relied on their surrounding water to support their daily living and recreations. Seafood was their primary source of dietary protein until strong influences from western countries. However, the per capita seafood consumption still exceeded global average. In 2015, per capita seafood consumption in Oceania was 25.0 kg/year vs 15.5 kg/year worldwide (FAO, 2018). Within Oceania, Pacific Small Island Countries (PICs) had an average national fish consumption ranging from 55 kg to 110 kg per person per year (Bell *et al.*, 2009).

Climate change and overfishing have threatened the sustainability of yield from capture fisheries in USAPI. Although there were some optimistic reports on future status of tuna stocks in the Pacific, foreign fishing companies have exported majority of their catch to consumers outside the islands. Islanders have relied on subsistence catch to meet their demand. As the harvest from nearshore fishery declined due to overfishing and pollutions, they have to consider secure their seafood supplies via aquaculture. In 2016, per capita seafood supply for Oceania was down to 24.3 kg while worldwide per capita supply increase to 19.7 kg (FAO, 2019).

USAPI has superior natural resources for aquaculture development, such as pristine water, year-round warm weather, and isolated condition for disease prevention. However, they have to overcome constraints to aquaculture development such as small land area, natural hazards for some islands, insufficient knowledge base, shortage of available capital, distant markets, and poor transportation systems. Other than natural hazards for some islands, the remaining constraints are solvable. Currently, the Pacific islands is still the least developed region in terms of aquaculture production worldwide with annual production around 24,091 tonnes or less than 0.022% of the total worldwide aquaculture production (FAO, 2019, www.fao.org/fishery/statistics/software/fishstati/en). A good strategic development plan is essential to reveal aquaculture potential in the region. With the complex political systems in the region, each island's entities must have their own development plan. In this region, there are two territories, one commonwealth and three independent countries.

This report will discuss the potential threat of climate change to fishery, review the status and challenges of aquaculture, and finally present some suggestions on future development.

Food Sources

Fish plays an important role in food security in USAPI. Traditionally, nearshore fishery is the main source of seafood for local consumption. Inhabitants were able to survive on the catch plus limited agriculture farming products from land. As distant fishery advanced, government leases out offshore fishing ground to foreign countries to harvest and export to outside of the countries. The fishing right leasing income played significant portion of total GDP in RMI and FSM (**Table 1**). Aids from foreign governments were another major income for the government (**Table 2**). As a result, communities have made a shift from eating traditional seafood items to importing cheap, processed foods. Consequently, many residents have suffered widespread health

Table 1. Fisheries Contribution

	FSM	Marshall	Palau	American Samoa	Guam	CNMI
FY2014 GDP						
Total$	318.1	186.7	249.08	711		
$	31.8	26.3	5.46	1.6	1.36	2.12
% of GDP	10.0	14.1	2.2	0.2		
200 mi EEZ (KM^2)	2,978,000	2,131,000	629,000	390,000	218,000	1,823,000
Per Capita Consumption (kg)	72.0-142.0	38.9-59.0	84.0-135.0	15.5	20.4-27.2	23.0

Table 2. Comparison of Top Donor Countries' and Organizations' Total Aid Spent in the Freely Associated States (2011–2018) (cited from Grossman *et al.*, 2019).

	United States	China	Australia	Japan	Taiwan	Multilateral Organizations
Federated States of Micronesia (FSM)	532.86	86.23	27.8	61.08		14.97 (World Bank)
Republic of the Marshall Islands (RMI)	313.6		31.23	48.9	51.99	16.7 (Asian Development Bank)
Palau	48.77		24.20	57.26	4.92	10.94 (Asian Development Bank)

Note: All figures are in U.S. dollars (million).

problems. Diabetes, cardiovascular diseases or hypertension significantly downgraded their quality of life (Carlin *et al.*, 2016; Charlton *et al.*, 2016). It is essential to revert their daily food diagram back to their tradition to regain their quality life. Increasing seafood supply via fish farming is one of the solutions to make seafood affordable and sustainable. Reliance on capture fishery as seafood supply is facing the challenge of climate change. Climate change may cause the migration of main tuna fisheries to other location (Bell *et al.*, 2016), thus, small island countries may lose both revenue and seafood. Losing seafood may mean more dependence on less healthy processed foods from abroad and increases medical expenses.

Climate Change and Fishery

USAPI has three main physiographical types: high volcanic islands, raised limestone islands, and low coral atolls and is the most vulnerable to climate change (Bell *et al.*, 2011). A few high islands in the region are large enough to influence local weather. The majorities are the small atolls and are at the mercy of sporadic rainfall for their fresh water. With the islands at or below sea level, rising sea level from global warming definitely imposes negative impacts on the livelihood of island habitants. The rising water temperature might also alter the distribution of tuna stock eastward from western part of the Pacific Ocean (**Fig. 1**; Bell *et al.*, 2016). Besides the redistribution of tropical tuna, global warming and ocean acidification can also cause damages to coral reef and other habitats for many aquatic species (Bell *et al.*, 2018). Bell *et al.* (2016) further predicted that the abundances of tuna would decrease due to the decline in richness of food web. To increase the resilience of USAPI to climate change, agencies such as US Agency for International Development (USAID) has created a Pacific-American Climate Fund to civil society organizations to reduce long-term vulnerabilities associated with climate change. Since the vulnerability is a function of potential impacts and adaptive capacity. Islanders have to strengthen their adaptive capacity to modify or change actual or expected climate stress to reduce the vulnerability and to stay at their islands.

USAID's Pacific-American Climate Fund has supported three aquaculture projects in Marshall Islands and Federal State of Micronesia to train local residents to produce seafood and aquatic products in their water and to generate income (Ellis *et al.*, 2018; Hicks and Murashige, 2018; Zackhras *et al.*, 2018). Although the scale is still small and not significant, it has potential to expand to a large scale and ultimately enhance the nature fish stocks via aquaculture.

Status of Aquaculture

Adam *et al.* (2001) presented the status of aquaculture in the Pacific islands. Now, the total production still did not show any noticeable increase after about twenty years. In 2017, the total aquaculture production worldwide reached 112 million metric tonnes (including aquatic plants) but

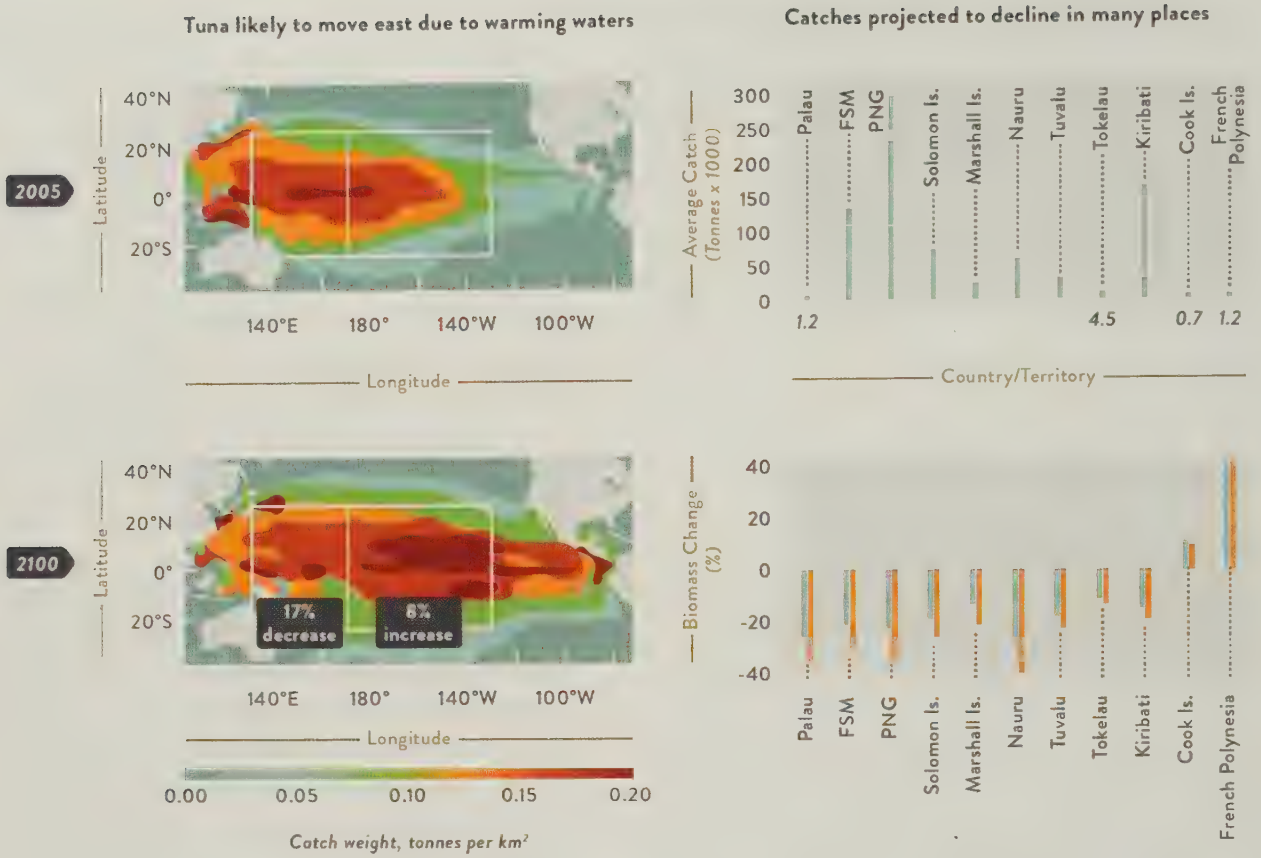

Fig. 1. Climate change and predict tuna distribution. From Bell *et al.* (2016).

the estimated contribution from USAIP was only at 201 metric tonnes (FAO, 2018). In spite of the lower productivity from aquaculture, aquaculture has been the focus of technical and development attention throughout the region. Numerous documents, reports and reviews on the accomplishments are available (Adams *et al.*, 2001). It shows the intention of developing aquaculture remains strong. Almost all island entities have their aquaculture development plan but did not completely implement for various reasons. Most of previous or on-going aquaculture projects led by outsiders have focused on technology transfer and capacity building. Projects have shown impressed outputs but not in actual production. Until now, the total aquaculture yield from USAPI was still insignificant and could not meet the daily domestic food demand. The followings are the highlight of regional aquaculture status.

Farming species- There are several target farming species in the region, which includes but not limit to giant clams, several hard and soft corals, marine ornamental fish, black pearl oyster, bath sponge, sea cucumber, Pacific threadfin (Moi), milkfish, rabbitfish, tilapia, groupers, marine shrimp, and mangrove crab, etc. (**Table 3**). Out of those farming species, giant clams, various coral species, ornamental fish, and processed bath sponge are export items currently. Sea cucumber and black pearl oysters are next two items with high export potential.

Research Facilities- There are aquaculture research facilities at University of Guam and community colleges throughout the region for research and training purpose. The following is a list of available facilities throughout the region.

1) Palau- Palau Mariculture and Demonstration Center (PMDC or giant clam hatchery) under Palau Marine Resources Bureau may be the most impressive governmental facility in the region. At junction to PMDC, Palau Aquaculture Center funded by Taiwan government is an upgraded marine finfish hatchery. Palau Community College runs a multi-species hatchery located at Ngermetengel Village, Ngeremlengui State. Biota Palau at Airai Old Dock Ordomel is a private operation and a branch of Biota

Table 3. List of Species Produced in U.S.-Affiliated Pacific Islands (USAPI)

Commonwealth of the Northern Mariana Islands (CNMI)	Tilapia, Marine shrimp, Rabbitfish
Guam	Tilapia, Marine shrimp, Milkfish, Freshwater prawn
Palau	Corals, Giant clams, Tilapia, Milkfish, Rabbitfish, Marine shrimp, Marine ornamental fish, Grouper
Federated States of Micronesia (FSM)	Corals, Sponges, Giant clams, Sandfish, Black pearl oyster
Republic of the Marshall Islands (RMI)	Corals, Giant clams, Pacific threadfin, Marine ornamental fish, Grouper
American Samoa	Tilapia, Giant clams

Florida. The company focuses on marine ornamental fish, coral, clams and other finfish species.

2) Guam- Guam Aquaculture Development and Training Center (GADTC), also known as the Fadian Hatchery, is under the care of the University of Guam (UOG) from 2001 until 2019. Now, a private company has established a lease agreement with UOG to operate the hatchery. The UOG Marine Laboratory can conduct basic and applied research on the biology of tropical marine organisms. Instead of aquaculture, the laboratory's research focuses on the conservation and development of marine resources of the near-shore waters of Guam and Micronesia.

3) FSM- In Pohnpei, College of Micronesia Land Grant Program has operated Nett Point hatchery since August 2001 for black pearl oyster and sea cucumber. This hatchery was installed in an old warehouse space next to water. At the east side of the island, a non-profit organization established Marine and Environmental Research Institute of Pohnpei works on several coral species, bath sponge, giant clam and ornamental species. In Kosrae, the Federated States of Micronesia National Aquaculture Center established in 1991 at Lelu produces giant clam, mangrove crab and others. In Chuuk, the Korea-South Pacific Ocean Research Center (KSORC) was established on 30 May 2000 on a small island, Weno Island, located within the Chuuk Lagoon with the long-term goal of promoting ocean research and related marine industries.

4) CNMI- College of North Mariana had a small aquaculture research facility but was destroyed by a recent hurricane in 2017. They have budget to rebuild another one.

5) RMI- College of Marshall Islands has one science station with facility for aquaculture research. Another finfish hatchery build by Rongelap Atoll local government under a grant from USAID has been used for research purpose as well.

Skilled workforce- USAPI has relied on foreign technicians to take full charge of farm operation and train local workers. College education and on-site training at funded aquaculture projects are two key sources for local aquaculture technicians. Local trained technicians have shown their abilities to operate black pearl oyster and sea cucumber hatcheries, for example. However, it seemed to be a challenge to assist those skillful workers to apply their technique in production. With less working opportunities, it has been a difficult task for them to find a good job. Often time those well-trained technicians had to find another different job after the funded projects ended.

Commercial farms- Because of the limitation of land area, over one hectare land-based grow-out farms only located in CNMI, Guam and Palau so far. Cage culture at open water so far only took place in Majuro, and Palau. Cage culture has great potential to expand to other islands where do not have natural hazards, such as hurricanes.

There were several commercial ventures taken place in USAPI. Currently, only a few operations remain. A hopeful commercial marine shrimp farm at CNMI was permanently stopped operation after strong hurricanes damaged the facilities in 2017. Marshall Islands mariculture farm with the strong support from parent company Ocean, Reef and Aquarium (ORA) in Florida is doing well in

ornamental fish and giant clams. The marine finfish cage farm by Rongelap Atoll local government in Majuro is progressing toward to be a commercial operation. In Palau, milkfish, rabbitfish and mangrove crabs are cultured for commercial purpose. Up to now, investors from outside have expressed interested in starting fish farming in USAPI but yet to be seen.

Path for the Future

Accepting USAPI is the most vulnerable to climate change and knowing coastal fisheries and staple food crops will decline due to climate change, skillful management of the harsh environment condition and nature resources is the only way to continually inhabit in USAPI for years to come. View the success or failure of aquaculture in the region while we review the status, we can conclude the pathway to the future success. USAPI has unique situation different from other countries in their social culture background. It indicates the essential need of community engagement in any aquaculture development. The initial aquaculture practice has to be simple technique and accessible to all required components. Any new practice should respect their traditional rural lifestyles and become a part of community social activities.

To utilize fully and sustainably the most valuable resource, *i.e.* EEZ, for food production, skillful workforce is the fundamental and essential base. Capacity building has been the center of all previous and on-going aquaculture projects. It seemed to be not an issue to find talents and train them. The big challenges are to retain skillful workforce. It is a fruitless effort of having skillful workforce but no established business ventures to receive them. After establishment of skillful workforce, appropriate farming technology can be developed and applied in food production. The appropriate farming technology should also consider the adaptation to the climate change (Wijkström, 2012). And, it is important to have a business mindset to implement any farming technology. We need the entrepreneurs connect all the above dots together, furthermore to the markets and attract capital for operation. Investors will be not interested unless they are able to determine which islands have friendly investment environments. A true partnership between private and public sectors is essential to make sure the sustainability of the development. Finally, everyone involved must have the "will" to overcome any threats in front of aquaculture development before we will be able to success (Lee and Awaya, 2003). The sustainable utilization of the unique natural resource at EEZ for fisheries development will have benefits to the people and economies of the Pacific region and extend far beyond their economic returns at every level.

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(1) Adams T., Bell J., and Labrosse P., 2001: Current status of aquaculture in the Pacific Islands, in "Aquaculture in the Third Millennium, Technical Proceedings of the Conference on Aquaculture in the Third Millennium" (ed. by Subasinghe R. P., Bueno P., Phillips M. J., Hough C., McGladdery S. E., and Arthur J. R.), NACA, Bangkok and FAO, Rome, pp.295-305.

In the book "Aquaculture in the Third Millennium" , this chapter "Current status of aquaculture in the Pacific Islands" gave an overview the status of aquaculture in the Pacific Islands at the turn of 20th century. It served as the base to assess any new development in 21st century. Through the new wave of international cooperation, it is expect to increase sustainable use of aquatic resources to meet the goal of food security. Food and Agriculture Organization of the UN (FAO) and Network of Aquaculture Centres in Asia-Pacific (NACA) co-organized this conference in 2000.

(2) Lee C. S., and Awaya K., 2003: Viable aquaculture development in the U.S. affiliated islands - lessons from giant clam and sponge farming. *Aquaculture Economics & Management*, **7(1&2)**, 125-135.

This paper reviewed the farming technology for giant clam and bath sponges. Then, it used giant clam and bath sponges as an example to

discuss the challenges of technology transfer, which include biological, technological, environmental, and socioeconomic, to Pacific Islands. Finally, some recommendation was made for the successful technology transfer.

(3) FAO, 2018: The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals, FAO, Rome, 227pp.

An important of aquaculture and fisheries status report published by FAO every two years. It highlights the critical importance of fisheries and aquaculture for the food, nutrition and employment of millions of people, many of whom struggle to maintain reasonable livelihoods. Data and graphics presented in this publication are widely used by research groups to assess the progress and to propose future works.

(4) Bell J. D., Johnson E. J., Ganachaud A. S., Gehrke P. C., Hobday A. J., Hoegh-Guldberg O., Le Borgne R., Lehodey P., Lough J. M., Pickering T., Pratchett M. S., and Waycott M., 2011: Vulnerability of Tropical Pacific Fisheries and Aquaculture to Climate Change: Summary for Pacific Island Countries and Territories, Secretariat of the Pacific Community, Noumea, New Caledonia, 386pp.

The book entitled *Vulnerability of Tropical Paciftc Fisheries and Aquaculture to Climate change* provides the region with the understanding needed, and the adaptations, policies and investments recommended to reduce the likely impacts of climate change on fisheries and aquaculture. It also gives the sector a roadmap for capitalising on the opportunities expected to arise from the changing climate. This book is the product of a partnership that started between the Australian Agency for International Development (AusAID) and the Secretariat of the the Pacific Community (SPC), and then grew to embrace contributions from 36 institutions. This summary, which is a companion to the book, provides this vital information in an accessible form for each Pacific Island country and territory. It is a quick access to understand projected changes to surface climate and the ocean, to oceanic fisheries, to coastal fisheries, to freshwater and estuarine fisheries, to aquaculture, economic and social implications, and adaptations and suggested policies. It is a valuable document to have a quick overview of potential climate change impacts and suggested remedy policy.

(5) Charlton K. E., Russell J., Gorman E., Hanich Q., Delisle A., Campbell B., and Bell J., 2016: Fish, food security and health in Pacific Island countries and territories: a systematic literature review. *BMC Public Health*, **16**, 285. (doi: 10.1186/s12889-016-2953-9)

This paper discussed the importance of fish to Pacific Island Countries and territories (PICTs) in both food security and health related concerns based on the review of 29 studies. However, there is a paucity of research aimed at assessing how maintaining and/or improving fish consumption benefits the diets and health of Pacific Islanders. Instead of fresh seafood, there is an increasing demand for packaged imported foods, such as canned meats, instant noodles, cereals, rice, and sugar-sweetened beverages, with subsequent decreased consumption of locally-produced plants and animals.

(6) Gillett R., and Tauati M. I., 2018: Fisheries of the Pacific Islands - Regional and national information, FAO Fisheries and Aquaculture Technical Paper No. 625, FAO, Apia, 401pp.

This paper discusses the important species, the status of the resources, and the fisheries management under offshore fishing and coastal (or nearshore) fishing. This report also provides information on the fisheries in each of the 14 independent Pacific Island countries (including Federated States of Micronesia, Marshall Islands, and Palau) in the following categories:

- Overview and main indicators
- Production sector
- Post-harvest sector
- Socio-economic contribution of the fishery sector
- Trends, issues and development
- Institutional framework
- Legal framework

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Towards effective coral community restoration for sustainable fishery of a coral reef grouper *Epinepherus ongus*: implications of ecosystem-based management

Atsushi NANAMI*

Abstract: Spawning aggregations of coral reef fishes are particularly vulnerable to fishing since only conspecific individuals gather at specific sites in restricted seasons and lunar phases. Since capturing spawning aggregations leads to significant negative impacts for both local stock and reproductive success, protection of spawning aggregations has been an urgent need recently for coral reefs all over the world. Furthermore, conservation of habitat in the non-spawning season is also essential for species that form spawning aggregations since habitat loss at their home ground would also lead to a reduction in their population. In the present paper, the focus is on a coral reef grouper species (*Epinephelus ongus*), that is a very important fishery target and forms spawning aggregations in an Okinawan coral reef. Effective coral community restoration is discussed to establish a sustainable fishery of the species. This author's previous studies have shown: (1) their home ground was several kilometers around the spawning ground; (2) their home range was very limited around a coral colony during non-spawning periods; (3) juveniles preferentially used corals with fine structure (*e.g.* bottle-brushed acroporid corals) whereas adults were mainly found at corals with coarse structure (*e.g.* massive corals and staghorn acroporid corals); and (4) very precise returning ability was observed after spawning migration (*i.e.* species would return to the coral colonies that were used before the spawning migration). These results suggest that: (1) conservation of the coral community around the spawning ground is indispensable; (2) coral species that are preferentially used by *E. ongus* should be selected for coral community restoration; (3) the area several kilometers around the spawning ground should be the proposed area for coral community restoration. Since the spawning ground of the species has already been assigned as a marine protected area, coral community restoration around the spawning ground would be useful to enhance the *E. ongus* stock.

Key words: coral community restoration, sustainable fishery, spawning aggregation, white-streaked grouper, *Epinephelus ongus*

Introduction

Habitat restoration is one of the essential tools for fisheries management, since almost all marine organisms require appropriate habitat space to complete their life cycle (*e.g.* settlement, growth and reproduction). Thus, an ecosystem-based management approach is needed. In coral reefs, numerous fisheries target species rely on the coral communities as their refuge space and resting site. However, recent coral degradation has caused significant negative impacts on populations of fisheries target species. Thus, coral community restoration has been urgently needed for sustainable fisheries in coral reefs.

Among the diverse fish species, at least 80 species have been reported to form spawning aggregations (Sadovy de Mitcheson and Colin, 2012). Domeier (2012) has defined reef fish spawning aggregations as consisting of only conspecific

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individuals where spawning is highly predictable in time and space. Spawning aggregations are usually found in restricted seasons and lunar phases at particular sites (Nemeth, 2009). Due to the ecological characteristics, spawning aggregations have great vulnerability to fishing (Sadovy de Mitcheson and Erisman, 2012). In addition, spawning aggregation fishing could decrease reproductive success of the species, leading to decreased larval supply and juvenile settlement. Thus, effective protection of spawning aggregations is needed for sustainable fisheries and reproductive success. Recently, marine protected areas have been applied to protect spawning aggregations all over the world.

However, if habitats around the spawning ground are degraded, the effects of spawning ground protection would decrease. This is because the species that form spawning aggregations inhabit their home ground around the spawning ground in the non-spawning periods. The habitat loss in the home ground would decrease population size of the species in the non-spawning periods and this would decrease the magnitude of spawning aggregations, even if the spawning aggregation is protected from fishing (**Fig. 1**). Thus, habitat restoration (*i.e.* coral community restoration) around spawning grounds should be considered as part of effective spawning aggregation protection. The protection of these restored habitats (*e.g.* marine protected areas) is an important additional step to ensure their longevity and success.

There are at least two aspects that determine success of coral community restoration: "How" and "Why". "How" means the techniques used to enhance survival rates or scale up coral communities. In contrast, "Why" is related to the purpose of restoration (*e.g.* biodiversity conservation or sustainable fishery). In the latter case, we should

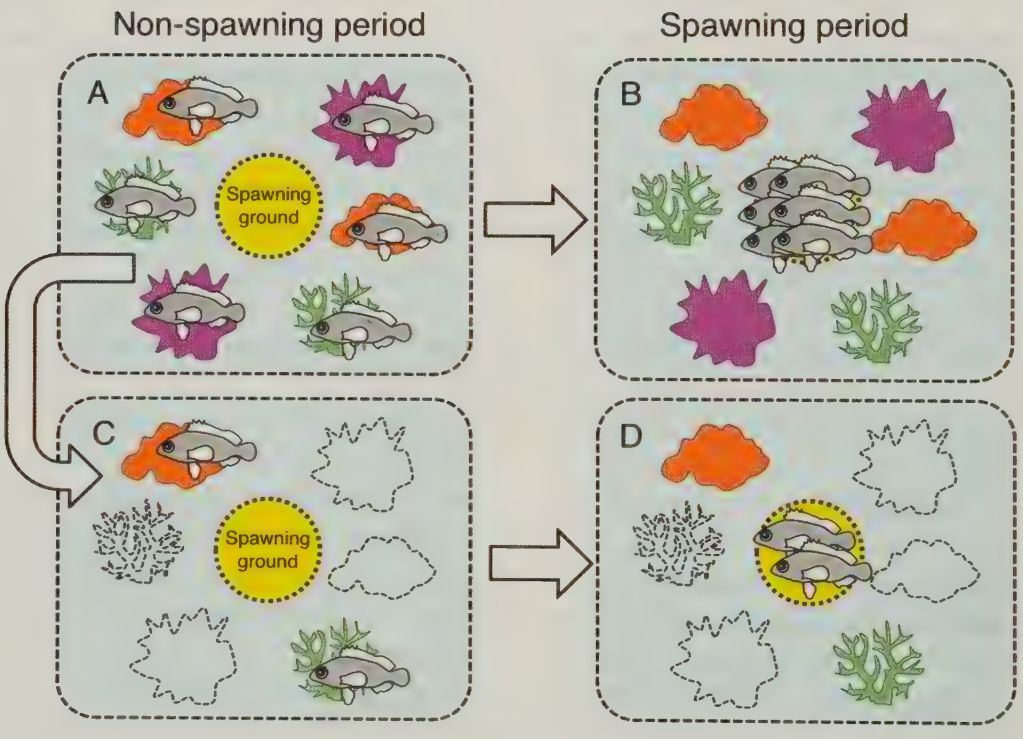

Fig. 1. Schematic diagram showing the reason why coral community should be conserved or restored around the spawning ground. If little damage to the coral community around the spawning ground and a large population size of the fish is found in a non-spawning season (A), then a large spawning aggregation would be found at the spawning ground during spawning season (B). If coral community degradation occurs around the spawning ground, the population size of the fish would decrease around the spawning ground (C), and only a small spawning aggregation would be found even if the spawning ground is protected from catch (D).

determine strategies such as "what is the target species to enhance?", "what types of corals should be restored?" and "which area is proposed for coral community restoration?" In order to determine the strategy, we should clarify ecological aspects for the target species or target communities before conducting coral community restoration. In the present paper, the focus is on a coral reef grouper in an Okinawan coral reef. Subsequently, the effective coral community restoration needed to achieve stock enhancement of the grouper is discussed.

Materials and Methods

Study species

White-streaked grouper *Epinephelus ongus* is one of the important fishery targets around the Okinawan region and is known to form spawning aggregations (Ohta and Ebisawa, 2015) (**Fig. 2A**). The spawning of the species was found to occur during the last-quarter moon in only one month (May) or two consecutive months (April-May or May-June). In order to protect the spawning aggregation of the species, the spawning ground has been protected during spawning periods since 2010 (Nanami *et al.*, 2017) (**Fig. 2B**).

In order to achieve effective spawning aggregation protection of the species, several ecological traits should be clarified such as (1) spawning migration distance, (2) microhabitat association and (3) returning ability after spawning migration of the species.

Definition of ecological terms

Four ecological terms are defined as follows (**Fig. 3**): (1) home ground: the area that are used by fishes in non-spawning period; (2) home range: the area that are used by a focal fish individual (*i.e.* territory); (3) spawning ground: the area that are used by fishes in spawning event; (4) microhabitat: the habitat that are associated by fishes in fine scale within several-tens centimeters (e.g. coral species and coral morphology). This is contrary to landscape-level habitat categorization (*e.g.* reef flat, reef crest and reef slope).

Spawning migration distance

Migration distance is defined as the distance between home ground and spawning ground for the focal individual. Clarification of the spawning migration distance would provide useful information to estimate the home ground area around the spawning ground. Namely, "how many areas should be considered for coral community restoration around the spawning ground?" Using a tag-and release method, the spawning migration distance was estimated. In total, 1157 *E. ongus* individuals were tagged and released at their home grounds in the non-spawning period and 350 were tagged at the protected spawning ground in the spawning period.

Microhabitat association

Understanding the microhabitat association would provide useful information to clarify what types of corals should be restored. Microhabitat associations

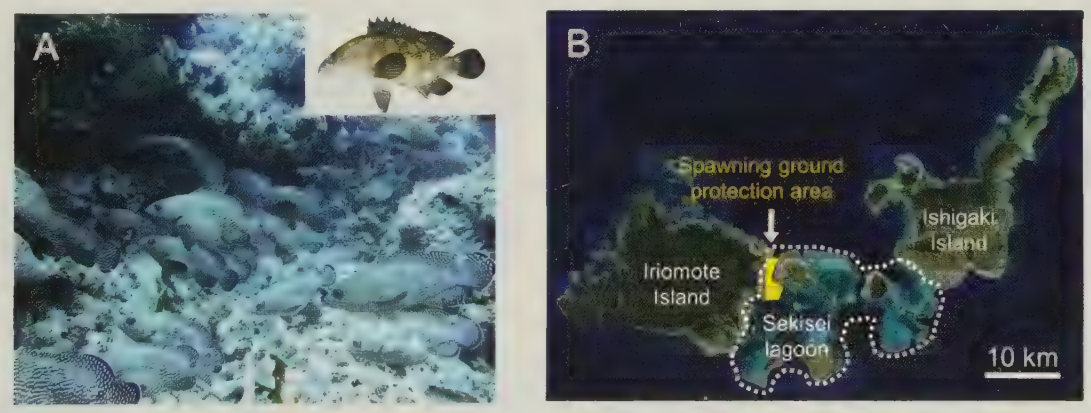

Fig. 2. Spawning aggregation of white-streaked grouper (A). Location of the spawning ground protection area in Sekisei lagoon, Okinawa, Japan (B). The aerial photograph was provided by the International Coral Reef Research and Monitoring Center.

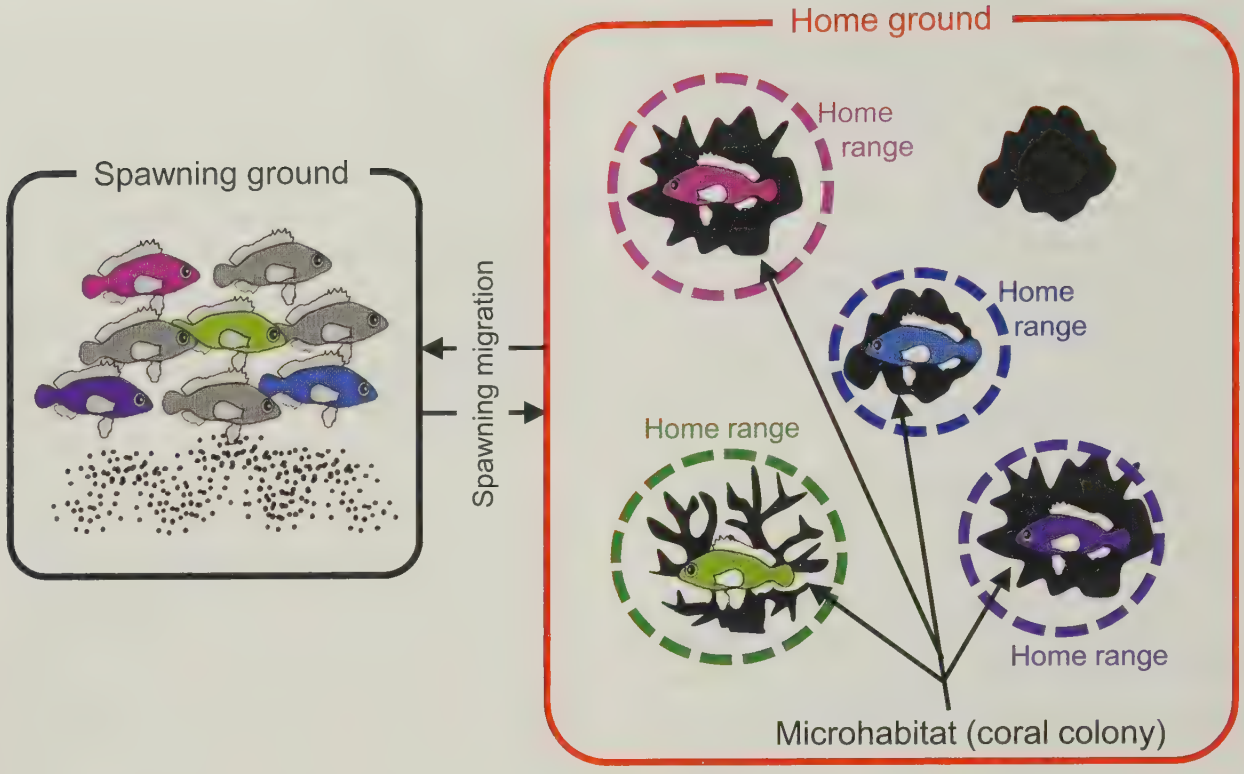

Fig. 3. Schematic diagram for four ecological terms (home ground, home range, spawning ground and microhabitat). (1) home ground: the area that are used by fishes in non-spawning period; (2) home range: the area that are used by a focal fish individual (*i.e.* territory); (3) spawning ground: the area that are used by fishes in spawning event; (4) microhabitat: the habitat that are associated by fishes in fine scale within several-tens centimeters (*e.g.* coral species and coral morphology).

for juvenile and adult white-streaked grouper were examined at home grounds by underwater observations.

Site fidelity and returning ability

Clarifying the site fidelity and returning ability would be useful to consider if the restored coral community would be used throughout the white-streaked grouper's lifetime. Seventeen individuals were captured and an acoustic coded transmitter was surgically implanted into the abdominal cavity of each fish. All tagged individuals were promptly released back to the site of their capture at the respective coral colony. In addition, 19 automated monitoring acoustic receivers were deployed at the release point. The location of each fish was detected using a VEMCO Positioning System.

Results

Migration distance

For the fish that were released at the home grounds, 23 individuals were recaptured at the spawning ground during the spawning periods (**Fig. 4A**). For the individuals that were released at the spawning ground, six individuals were recaptured outside the spawning ground (**Fig. 4B**) (Nanami *et al.*, 2015). The estimated migration distances from the home ground to the spawning ground ranged from 2.2 to 8.8 km (**Fig. 4C**).

Microhabitat association

Most juveniles (total length < 14 cm) were found in corals with fine structure (*e.g.* bottlebrush *Acropora* and arborescent *Acropora*), whereas most adults (total length > 18 cm) were found in corals with coarse structure (*e.g.* staghorn *Acropora*, massive *Porites* and branching *Porites*) (**Fig. 5**) (Nanami *et al.*, 2013; Nanami unpublished data).

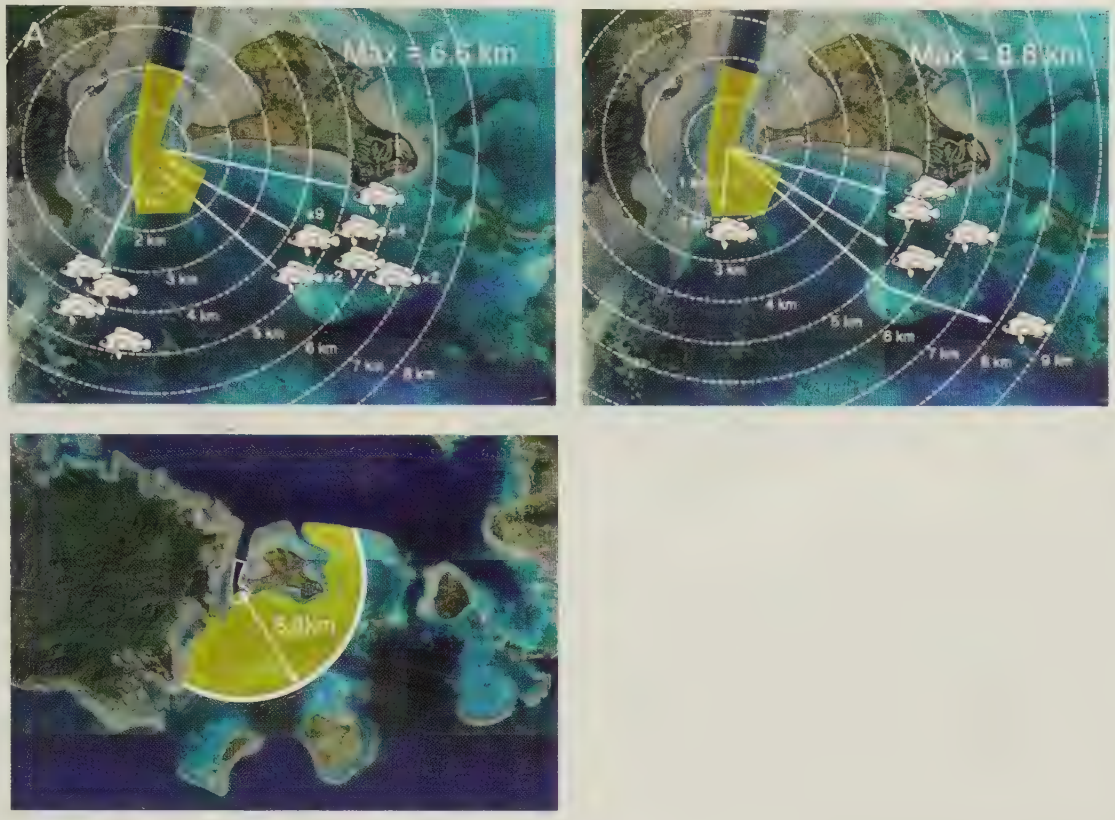

Fig. 4. Estimation of spawning migration distance by tag-and-release. Results of 23 individuals released outside the spawning ground (A) and 6 individuals released inside the spawning aggregation (B). The estimated home ground of white-streaked grouper around the protected spawning ground (C). Redrawn from Nanami *et al.* (2015). The aerial photographs were provided by the International Coral Reef Research and Monitoring Center.

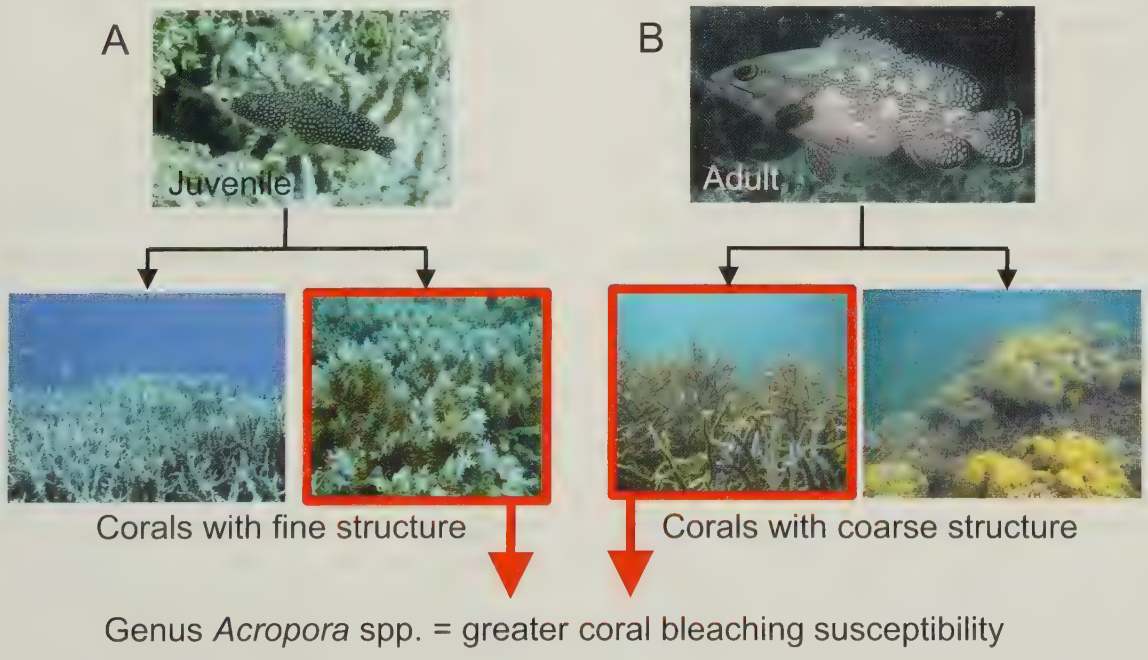

Fig. 5. Microhabitat association of juveniles (A) and adults (B) of the white-streaked grouper. Both fine-structure and coarse-structure corals included genus *Acropora*, which has greater coral bleaching susceptibility and subsequent death.

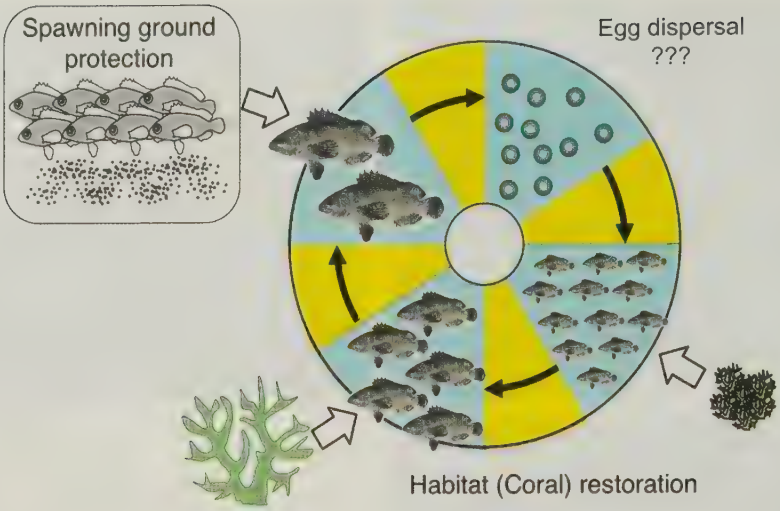

Fig. 6. Schematic diagram showing the strategy to enhance spawning aggregation protection by coral community restoration. Since the spawning ground has already been protected, other protective actions at earlier life stages would be effective. If appropriate, corals are selected and when these coral communities are successfully restored, fish population size would increase at their home ground resulting in large spawning aggregations at the protected spawning ground during the spawning season. The manner in which egg dispersal occurs should be clarified in the future.

Site fidelity and returning ability

Tagged individuals showed high site fidelity to their release point (patchy coral substrates). For returning ability, 10 individuals were analyzed and 8 out of the 10 individuals showed precise returning after the spawning migration to the patchy coral substrates that were used before the spawning migration (Nanami *et al.*, 2018).

Discussion

The results of the present study showed several strategies for coral restoration to enhance spawning aggregation protection (**Fig. 6**): (1) conservation of coral communities around the spawning ground is indispensable; (2) coral species that are preferentially used by *E. ongus* should be selected for coral community restoration. In particular, restoration of genus *Acropora* is urgently needed due to the greater susceptibility to coral bleaching; (3) the area that is c.a. 8.8 kilometers around the spawning ground should be the proposed area for coral community restoration. Since high site fidelity and returning ability after spawning migration were found, it follows that the successfully restored coral communities would be used by the species throughout their lifetime. One of the future challenges is to clarify the manner of egg dispersal from the protected spawning ground (*e.g.* Almany *et al.*, 2013). If the eggs produced from the protected ground arrive at a restored coral community, it is expected that greater settlement at the restored corals would be exhibited.

Acknowledgments

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Nanami A., Ohta T., and Sato T., 2015: Estimation of spawning migration distance of the white-streaked grouper (*Epinephelus ongus*) in an Okinawan coral reef system using conventional tag-and-release. *Environ. Biol. Fish.*, **98**, 1387-1397.

Nanami A., Sato T., Kawabata Y., and Okuyama J., 2017: Spawning aggregation of white-streaked grouper *Epinephelus ongus*: spatial distribution and annual variation in the fish density within a spawning ground., *PeerJ*, **5**, e3000.

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Nemeth R. S., 2009: Dynamics of reef fish and decapod crustacean spawning aggregations: underlying mechanisms, habitat linkages, and trophic interactions, in "Ecological connectivity among tropical coastal ecosystems" (ed. by Nagelkerken I.), Springer, Dordrecht, pp.73-134.

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This study examined the dispersal distance of larvae from the spawning ground for a coral reef grouper, *Plectropomus areolatus*. The parentage analysis revealed that 50% of larvae settled within 14 km of the spawning ground. The noteworthy point of the study is that spawning ground protection would be effective at a local scale (within several to ten kilometers) for larval dispersal.

(2) Nanami A., Sato T., Takebe T., Teruya K., and Soyano K., 2013: Microhabitat association in white-streaked grouper *Epinephelus ongus*: importance of *Acropora* spp.. *Mar. Biol.*, **160**, 1511-1517.

This study quantified microhabitat associations for juvenile and adult white-streaked grouper. For juveniles, most individuals showed a significant positive use of bottlebrush *Acropora*. For adults, most individuals showed a significant positive use of staghorn *Acropora*. A habitat choice experiment, using pre-settlement individuals, revealed that both bottlebrush *Acropora* and staghorn *Acropora* were used as settlement sites, whereas coral rubble was rarely used as a settlement site.

(3) Nanami A., Ohta T., and Sato T., 2015: Estimation of spawning migration distance of the white-streaked grouper (*Epinephelus ongus*) in an Okinawan coral reef system using conventional tag-and-release. *Environ. Biol. Fish.*, **98**, 1387-1397.

Using a tag and release method, this study estimated the migration distance and the degree of unified movement of white-streaked grouper associated with the spawning migration. In total, 1157 *E. ongus* individuals were tagged and released

at their home grounds in the non-spawning period and 350 were tagged at a known spawning ground in the spawning period. The estimated migration distances from the home ground to the spawning ground ranged from 2.2 to 8.8 km.

(4) Nanami A., Sato T., Kawabata Y.. and Okuyama J., 2017: Spawning aggregation of white-streaked grouper *Epinephelus ongus*: spatial distribution and annual variation in the fish density within a spawning ground. *PeerJ*, **5**, e3000.

This study revealed 1) spatial variations in the density of *E. ongus* at the spawning ground, 2) the relationship between fish density and environmental variables, 3) inter-annual variations in the spawning aggregation, 4) the proportion of males to females at the spawning ground for several days pre-and post-spawning and 5) the relationship between male density and female density at the protected spawning ground, based on observations over five years at an Okinawan coral reef.

(5) Nanami A., Mitamura H., Sato T., Yamaguchi T., Yamamoto K., Kawabe R., Soyano K., Arai N., and Kawabata Y., 2018: Diel variation in home range size and precise returning ability after spawning migration of a coral reef grouper *Epinephelus ongus*: implications for effective marine protected area design. *Mar. Ecol. Prog. Ser.*, **606**, 119–132.

This study examined the diel variation in home range size and the degree of precision for returning ability of white-streaked grouper by acoustic telemetry. Seventeen individuals were studied, and nighttime home range sizes were over 5-times greater than the respective daytime home ranges. Returning ability for 10 individuals that showed clear spawning migration behaviour was also analyzed and 8 out of the 10 individuals showed precise returning after the spawning migration to the patchy coral substrates that were used before the spawning migration.

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Scaling up coral restoration to meet the demands of a collapsing ecosystem

Tali VARDI*

Abstract: Coral reefs feed millions of people on earth and are critical to the livelihoods of millions more. They house approximately a quarter of the ocean's biodiversity and are critical to fisheries worldwide. Their importance and fragility to humans cannot be overstated. According to climate models, in about thirty years half of all coral reefs are predicted to disappear due to bleaching caused by unprecedented ocean warming (Bindoff *et al.*, 2019). If political and economic forces begin to seriously address emissions, corals will still need help maintaining enough abundance and diversity to rebuild reefs by the end of the century when ocean temperatures may begin to stabilize. With the tacit assumption that emissions will be curbed, an explosion of coral restoration and research into coral "interventions has taken place over the past two years. Coral aquaculture is a pivot point of any advancement in scaling-up restoration or implementing a new intervention – as any and all applications involve the delicate care and propagation of corals in the water or on land. NOAA has taken a lead role in coordinating coral restoration efforts throughout the US and the globe, primarily via the Coral Restoration Consortium and by commissioning the National Academies of Sciences to review coral interventions. This paper highlights recent restoration and intervention successes in the U.S. and globally, and describes NOAA's proposed research and action plan on coral interventions.

Key words: coral, restoration, larval propagation, coral restoration, coral bleaching

Background

Coral reef restoration is a new and rapidly evolving field, made necessary by the calamitous degradation of coral reefs worldwide (Bostrom-Einarsson *et al.*, 2020). The primary threat to coral reef ecosystems is ocean warming, and current climate change trajectories which predict a rise in global temperature of 2°C, also predict a loss of 99% of the world's reefs (Bindoff *et al.*, 2019). The increasing severity and frequency of climate-change related coral bleaching events and tropical storms are limiting the ability of coral reefs to recover naturally between disturbances (Fabricius *et al.*, 2017; Hughes *et al.*, 2019). With this rapidly changing, unprecedented scale of decline, there has been an increasing shift in management priorities from a reliance on passive interventions that facilitate natural recovery processes (*e.g.* marine protected areas) to active interventions (*e.g.* coral gardening) which promote adaptation and resilience to changing conditions (Rinkevich 2005, 2008, 2019; Young *et al.*, 2012; Possingham *et al.*, 2015; Bostrom-Einarsson *et al.*, 2020).

The Coral Restoration Consortium

As the field of coral reef restoration and the number of practitioners grows rapidly, so does the need to share the successes and failures, and to ensure the latest science reaches restoration practitioners working underwater. To meet this need, the Coral Restoration Consortium (CRC) was started by NOAA in 2017 to facilitate cooperation and communication among coral reef restoration practitioners, managers, researchers, and educators

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in order to support scientific and practical ingenuity in the field. At its inception the CRC focused on the Caribbean and most of the leadership was from the Caribbean. Within the past year, as reefs have struggled and a growing number of locations engage in restoration, the CRC has expanded globally. In December 2018, the CRC hosted the first ever international conference on coral reef restoration and shortly afterwards voted in new Steering Committee Members from diverse tropical regions. In September 2019, we adopted three Regional Groups (Eastern Tropical Pacific, Latin America, and Australia). Japan is a notable exception and we would welcome a Japanese Regional Group!

Priorities for Coral Restoration

Here we present the six priorities as defined by Coral Restoration Consortium to help guide and promote the scaling-up of coral reef restoration efforts across disciplines and localities. These priorities were not developed to the exclusion of the priorities developed by other organizations. The task of conserving and restoring coral reefs is immense. No one organization nor a consortium of organizations will solve these issues alone. Our goal in articulating these priorities is to encourage others to join, either formally or via parallel efforts, so that we are working towards common goals.

1. Increase restoration efficiency, focusing on scale and cost-effectiveness of deployment.

A large majority of coral reef restoration projects fall under the "coral gardening" umbrella (Bostrom-Einarsson *et al.*, 2020). This technique involves taking coral fragments (often grown in a nursery) and transplanting them onto a degraded reef. The CRC takes pride in tackling restoration issues from multiple angles. While the CRC provides guidance based on the latest science, we also have a firm understanding that most of the restoration occurring in the world today is the relatively simple technique of coral gardening. Although this technique alone will not save the world's coral reefs – it can be done more or less effectively, and more or less efficiently. By improving the efficiency of the most common method, we could vastly increase the potential for corals to weather the next 50–100 years.

2. Scale-up larval propagation for its effective integration in coral reef restoration efforts, with an emphasis on recruit health, growth, and survival.

Larval recruitment from sexually assisted coral reef restoration is essential for improving the genetic diversity of small coral populations. Recently, important advances have been made in coral in vitro fertilization, as well as husbandry of coral larvae (reviewed in Randall *et al.*, 2020). However, larval propagation research has focused on broadcast spawning species and post-settlement mortality continues to be high. To increase the scale and efficiency of larval propagation we need to expand the use of existing larval propagation technologies (more species, more places), develop new technologies, and develop criteria for maximizing genetic diversity.

3. Develop guidance that promotes a holistic approach to coral reef ecosystem restoration.

The CRC is developing a roadmap to help the restoration community evolve from restoring single species of corals to restoring a functioning coral reef ecosystem that will be resilient to current and future biophysical challenges.

4. Develop guidance to ensure restoration of threatened coral species takes place within a comprehensive population genetics management context.

In many places around the world, critical reef-building corals are suffering population declines requiring strategic and thoughtful population management plans that can allow these species to be used effectively in restoration programs. To do this, careful consideration is needed to maximize any remaining genetic diversity of degraded coral populations to allow for successful sexual reproduction, adaptation, and recovery (Baums *et al.*, 2019). Sound guidance is needed to ensure population management plans are developed with appropriate genetic (including epigenetic), propagation, husbandry, and environmental considerations, and an understanding of new reef states (Rinkevich, 2020).

5. Develop and promote the use of standardized terms and metrics for coral reef restoration.

Although terrestrial habitat restoration has been conducted for decades, coral reef restoration is a relatively new field. A lack of common definitions and standardized metrics slows the transmission of ideas and the adoption of new techniques, while limiting comparability across projects (Bostrom-Einsarsson *et al.*, 2020). To increase the scale and efficiency of coral reef restoration, we must be able to communicate effectively across regions, fields of study, and public and private sector stakeholder groups with varying levels of technical expertise. This entails (1) identifying commonly used terms, comparing existing definitions, and establishing standard definitions when possible (while also acknowledging differing or conflicting definitions); and (2) developing and promulgating standard metrics for evaluating the success of coral reef restoration efforts.

6. Develop new and synthesize existing resources to guide and support coral reef restoration practitioners working in diverse geographic locations.

While there is a clear need to increase the efficiency of current methods, develop new techniques and approaches, and formalize the best available science, none of this has utility if the information does not reach the hands of people doing the work. Practitioners need knowledge exchanges, resources to build capacity, and access and training to new technological tools.

Recent Accomplishments of the Coral Restoration Consortium

Most of the work that the Coral Restoration Consortium does is via its Working Groups. Here I will outline a few recent accomplishments. The Monitoring Working Group developed a robust webinar on Photomosaics in coordination with the Reef Resilience Network. The webinar presents the state of the science, the benefits of monitoring reefs with this technology, low-budget as well as high-resolution options for implementation, and resources for adoption. Photo-mosaics are an ideal method for documenting the successes and challenges of reef restoration. The large area images can illustrate the ultimate metric of reef health at an appropriate ecological scale. They document species composition, coral cover, and growth of corals, and allow a re-examination of past data. The webinar relied on expertise from several academics and featured case studies by two reef restoration practitioners. It is archived on the Reef Resilience website. The Monitoring Working Group has also prepared Coral Restoration Monitoring Guidelines (Goergen *et al.*, 2020) as well as a beta version of a Restoration Database.

The Genetics Working Group published two recent papers: Molecular tools for coral reef restoration: beyond biomarker discovery (Parkinson *et al.*, 2020), and Considerations for maximizing the adaptive potential of restored coral populations in the western Atlantic (Baums *et al.*, 2019). The latter is a succinct guide to maximizing genetic diversity using various coral aquaculture techniques for restoration. Despite differences in species and environmental conditions, restoration is happening throughout the globe. Although this paper uses a Caribbean example, the recommendations serve as a helpful starting point for coral aquaculture and restoration elsewhere. The paper explicitly recommends the number of genets per area that should be planted to maintain or maximize diversity. Because bleaching, coral gardening, and assisted evolution have the potential to reduce genetic diversity, having clear guidelines that managers and practitioners can understand is paramount for proper aquaculture and coral reef restoration techniques.

Members of the Land-based and Larval Propagation Working Groups created the first trans-regional crosses of an endangered coral population using cryopreservation (Hagedorn *et al.*, 2018). Rescuing genetically depauperate coral populations by increasing genetic diversity from nearby populations, is one of the most feasible coral interventions proposed. This study demonstrated for the first time that viable juveniles of an endangered coral can be created by artificially inseminating cryo-preserved sperm from one population, with eggs of another regionally-distinct population. This project highlighted the importance and need for additional

expertise in coral larval rearing and early life-stage aquaculture. Currently, there are only a handful of such experts worldwide. As corals continue to be threatened with extinction primarily due to climate change, these techniques will become increasingly necessary for the long-term persistence of coral reefs.

US National Academies Review of Coral Interventions

In 2018, NOAA commissioned a two-part study by the U.S. National Academies of Sciences, Engineering, and Medicine to examine ecological and genetic interventions that have the potential to increase the resilience of coral reefs. Most of these techniques rely on coral aquaculture in one form or another. The *Research Review* describes the benefits, potential scale of application, current feasibility, risks, limitations, and infrastructure needs for 23 intervention types (NASEM, 2019a). The *Decision Framework* outlines an adaptive management strategy by which interventions can be evaluated against each other, additional research needs, and recommendations for the Caribbean (NASEM, 2019b). Research on coral interventions is progressing very rapidly and these reports thoroughly capture the current state of the science and make recommendations about how the field should move forward in the near future. Given the accelerating pace of threats to reef ecosystems, it is clear that the coral conservation community will need effective, timely interventions, judiciously applied; and that coordinated bodies of scientists, governmental officials, and other stakeholders, will have to decide which blend of conventional management and interventions will maximize their local reef's ability to persist considering budget, local buy-in and policy.

Based on these reports, NOAA created an action plan to guide how the agency will approach coral interventions in the next one to three years (Vardi *et al.*, 2020). Four primary actions are identified: (1) research and test priority interventions (2) develop local or regional structured decision support. (3) review policy implications of coral interventions. (4) invest in infrastructure, research, and coordinate global efforts to maximize results. Under Action (1), the interventions that NOAA chose to prioritize fit the following six objectives (taken directly from Vardi *et al.*, 2020):

1. Increase Diversity of Coral Populations. Coral populations are becoming increasingly small and fragmented, leading to depensatory effects that further limit spawning and recruitment. Increasing the stress tolerance or simply the genetic diversity of small, fragmented populations by importing corals from populations in different parts of the species' range or even from within a population, may be some of the least risky and most effective intervention strategies. The interventions that would help accomplish this objective are: Assisted Gene Flow, Outcross Between Populations, Supportive Breeding, and Cryopreservation.
2. Improve techniques to support interventions. Techniques to support interventions include: identifying stress tolerant coral colonies or genes ("Managed Selection"), expanding cryopreservation capabilities to capture current genetic variation for future research and restoration, and harnessing the diversity and abundance of coral spawning. Gamete and larval capture and seeding research is being led by institutions in Japan, Australia, and SECORE international.
3. Develop a framework for coral epidemiology. Disease is ravaging Caribbean corals and worsening climate conditions are likely to increase the frequency and severity of coral disease outbreaks worldwide. The research, veterinarian, management, and restoration community needs an epidemiology framework for coral-disease intervention, as well as research and development of therapies and delivery mechanisms.
4. Stress-harden corals ("Pre-exposure"). Multiple lab experiments and field observation have demonstrated that corals can increase their resilience to temperature and ocean acidification stress under certain conditions.
5. Manipulate algal symbionts to improve thermal tolerance ("Algal Symbiont Manipulations").

Coral bleaching is expected to increase in frequency over the next decades. Algal symbionts vary in their thermotolerance. Interventions that take advantage of this variability have experimentally increased the thermotolerance of the coral holobiont.

6. **Assess feasibility of environmental interventions.** Environmental interventions are manipulations to the physical or chemical environment to reduce or prevent bleaching, or reduce acidification. They can be geared to protect high-value sites such as nurseries or frequent tourist destinations. Examples include "Shading" corals from incident light, "Mixing of Cool Water", and changing the alkalinity of reef waters by restoring nearby plant communities ("Seagrass Meadows and Macroalgal Beds").

Conclusion

Due to precipitous ocean warming, restoration (including intervention) is a necessary bridge to stave the demise of tropical coral reef ecosystems. Here I have described how NOAA and the Coral Restoration Consortium are approaching this topic. However, the following cannot be overstated – these interventions are fruitless without addressing basic local and regional reef conditions such as sedimentation, eutrophication, and over-fishing as well as global climate change. Restoration should always be conducted within broader resilience-based management strategies in coral reef ecosystems and ideally within effectively managed marine protected areas that reduce and control background factors of coral reef degradation. Coral reef protection and restoration requires local management efforts as well as climate change mitigation if we want to witness coral reef recovery and the restoration of reef ecosystem services.

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NOAA commissioned the U.S. National Academies to review the science behind next generation coral restoration techniques. Most of these techniques rely on coral aquaculture in one form or another. The Research Review groups 23 interventions types (*e.g.* assisted evolution, marine shading to prevent bleaching, genetic engineering) into four categories and describes the benefits, potential scale of application, current feasibility, risks, limitations, and infrastructure needs for each. The Decision Framework outlines an adaptive management strategy by which interventions can be evaluated against each other, additional research needs, and recommendations for the Caribbean. Research on coral interventions is progressing very rapidly and these reports thoroughly capture the current state of the science and make some solid recommendations about how the field should move forward in the near future.

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Rescuing genetically depauperate coral populations by increasing genetic diversity from nearby populations, is one of the most feasible coral interventions proposed. This study demonstrated for the first time that viable juveniles of an endangered coral can be created by artificially inseminating cryopreserved sperm from one population, with eggs of another regionally-distinct population. This project highlighted the importance and need for additional expertise in coral larval rearing and early life-stage aquaculture. Currently, there are only a handful of such experts worldwide. As corals continue to be threatened with extinction primarily due to climate change, these techniques will become increasingly necessary for the long-term persistence of coral reefs which feed hundreds of millions of people and provide habitat to 25% of marine fisheries globally.

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Data from more than 2500 reefs across the Indo-Pacific Ocean were analyzed to delineate key drivers and suggest appropriate management at three different levels: protection, recovery, and transformation. Over 50% of surveyed reefs would benefit from recovery which includes some form of restoration in addition to traditional management, and, most importantly, reduction in ocean warming. Using comprehensive studies like this and the Global Coral Reef Monitoring Network's reports, should help nations prioritize coral research, restoration, and management efforts.

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This paper is a succinct guide to maximizing genetic diversity using various coral aquaculture techniques for restoration. Despite differences in species and environmental conditions, restoration is happening throughout the globe. Although this paper uses a Caribbean example, the recommendations serve as a helpful starting point for coral aquaculture and restoration elsewhere. The paper explicitly recommends the number of genets per area that should be planted to maintain or maximize diversity. Because bleaching, coral gardening, and assisted evolution have the potential to reduce genetic diversity, having clear guidelines that managers and practitioners can understand is paramount for proper aquaculture and coral reef restoration techniques.

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Sustainable large-scale coral restoration by establishing "artificial spawning hotspots"

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Abstract: Coral reefs have degraded over time and, recently, severe bleaching events caused further damage to coral communities worldwide from 2015 to 2017. Because coral reefs are an important habitat for coastal fish and invertebrates in tropical coasts, the decline of coral reefs also results in decreased fishery productivity. Therefore, for sustainable use of these resources, it is important to conserve coral communities that act as fishing grounds and fish nurseries. Coral transplantation is known as an effective restoration method. However, large-scale transplantation requires a great deal of labor and the transplanted corals are vulnerable to extermination by only a single disturbance such as bleaching or an outbreak of crown-of-thorns starfish (COTS). Hence, enhanced annual coral larval recruitment is needed for sustainable large-scale restoration.

Two key factors are crucial for sustainable coral restoration by enhancing reproduction. The first factor is establishment and maintenance of "artificial spawning hotspots" that consist of densely populated conspecific adult colonies. The second factor is improvement of early life survivability by collecting eggs and sperms at spawning and rearing larvae until settlement. For "artificial spawning hotspots," safeguards are required against predation by COTS and the use of shading is desirable against bleaching during the high seawater temperature season. In addition, a special gamete and larval collector, termed "larval cradle", was developed to consistently perform bundle collection, fertilization, and larval rearing. Eventually, it is suggested that a set of simple methods and techniques are used for sustainable large-scale coral restoration.

Key words: larval supply, *Acropora*, fish nursery, larval cradle, artificial substrate

Current Coral Reefs Status and Crisis

Coral reefs are large biogenic structures that develop in shallow waters in tropical and subtropical regions. Most of the modern coral reefs have formed atop stacks of dead corals created several thousand years ago (Braithwaite *et al.*, 2000; Kayanne *et al.*, 2002; Hongo and Kayanne, 2009). Recently, several disturbance factors frequently endanger coral reefs (De'ath *et al.*, 2012). One of those factors is coral bleaching. Corals have symbiotic microalgae, called "zooxanthellae", which largely contribute to coral growth by providing nutrients obtained by photosynthesis to the host coral (Tanaka *et al.*, 2018). This symbiotic relationship can collapse due to high seawater temperature where corals lose symbiotic algae (Fujise *et al.*, 2014). Such corals become white in color and this phenomenon is called "bleaching." Some researchers have described bleaching of corals as an adaptive behavior to environmental change, known as "adaptive bleaching" (Kinzie *et al.*, 2001). In any case, if the ambient temperature is 1℃ higher than normal for longer than 1 month, many corals die after bleaching. Some taxonomic groups such as genus *Acropora*, *Montipora*, and *Seriatopora* are susceptible to bleaching (Marshall and Baird, 2000), though these groups still dominate most of the Indo-Pacific reefs. Global warming may cause an increase in seawater temperature and some predictions forecast that the frequency of large-scale bleaching

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events will be higher than in past centuries.

Another crisis is outbreak of crown of thorns starfish (COTS) (Moran *et al.*, 1992). COTS is a large starfish armed with poisonous spines that predates corals, especially *Acropora* and *Montipora* species (Pratchett *et al.*, 2009). Generally, the population density of COTS is 1–2 individuals per 100 m^2. Once an outbreak occurs, the density increases by 100 times, and several hectares of corals can be eaten up within a few months (Suzuki *et al.*, 2012). Factors that cause a COTS outbreak are not clear, but it is probable that high nutrients contribute to outbreaks because COTS larval survival was drastically improved by increased phytoplankton density (*i.e.* the prey of COTS larvae) that resulted from high nutrient concentrations in laboratory experiments (nutrient hypothesis) (Fabricius *et al.*, 2010).

The third crisis is physical damage to corals caused by large typhoons (cyclones or hurricanes). Some corals that have weak skeletal structures, such as branching corals, are easily and severely damaged by large typhoons that typically occur once every several years.

It is important to understand the extent of anthropogenic impacts on coral reef destruction. For bleaching, if the cause of global warming is the accumulation of greenhouse gases such as CO_2 primarily discharged by human activities, bleaching is thus a consequence of anthropogenic activities. However, it is difficult to distinguish the roles between global warming and the natural cycle of the global environment (*e.g.* the frequency of El Niño) on the occurrence of bleaching (Claar *et al.*, 2018). In addition, it is probable that eutrophication and/or increase of bacteria in the water column accelerate damage to corals during bleaching (Fabricius *et al.*, 2013). For outbreak of COTS, if the nutrient hypothesis as stated above is true, the frequency of COTS outbreak is expected to increase due to anthropogenic impact. Similarly, anthropogenic impacts may indirectly increase the size of typhoons because global warming may be a contributing cause (Balaguru *et al.*, 2016). Although the extent of anthropogenic impacts on these crises remains poorly understood, coral populations are seriously damaged by a higher frequency of disturbances, especially in the long term, because they decrease coral reproductive output.

As mentioned above, most disturbances have large impacts on specific taxa such as *Acropora*. Although *Acropora* species are fast growing corals, it still takes at least 3 years (5 years on average) to grow to a mature size. Furthermore, the older the corals grow, the more eggs they spawn that contribute to larval supply. If most corals die from each disturbance within 10 years, robust reproduction (*i.e.* adequate larval supply) lasts only a few years and this could also gradually reduce coral populations.

Impact on Fisheries Resources

Fisheries resources in coral reefs have been a primary source of protein for people in tropical regions (Burke *et al.*, 2011). Many fish and invertebrates directly or indirectly depend on coral communities. For example, juveniles of one of the dominant groupers, *Epinephelus ongus*, selectively settle in the bottle-brush coral (*Acropora* species) habitat (Nanami *et al.*, ,2013). Most parrotfishes utilize the space among coral branches (mainly branching *Acropora* species) as a sleeping bed and coral recruitment is enhanced by fish grazing (Russ *et al.*, 2015). Some cuttlefish lay eggs in the space among coral branches (mainly branching *Porites* species). In addition, small fish such as damselfish and cardinalfish live associated with corals, which feed large fish such as groupers (Shpigel and Fishelson, 1989; Nakai *et al.*, 2001). Importantly, recent studies suggested that organic matter produced by symbiotic algae through photosynthesis contributed to the whole coral reef ecosystem as primary production. Therefore, it is highly probable that the decrease of corals is linked to the loss of fisheries resources (Wilson *et al.*, 2006). In terms of reproduction, "spawning aggregation" is one of the representative behaviors in the reproduction for some reef fishes such as groupers (Domeier and Colin, 1997; Nanami *et al*, 2017). Considering that such behavior could also be affected by the decrease of corals, the loss of fisheries resources is likely very serious with long-term consequences.

Previous Studies on Coral Restoration

Corals reproduce sexually and asexually. "Fragmentation" is a process of asexual reproduction, in which some branches of corals regrow as a new colony by adhering to the seafloor once they snapped off. Corals have various methods of sexual reproduction including mass spawning (both gonochoric and hermaphroditic) and larval brooding (Baird *et al.*, 2009). Corals produce swimming planula larvae that settle on the seafloor and metamorphose to juvenile corals.

One of the simplest methods for coral restoration is transplantation of coral fragments that can be accomplished using different techniques such as direct attachment of coral fragments (fallen branches are more effective) onto the seafloor, or attachment on artificial plates in aquaria followed by fixation on the seafloor. The first report of the coral fragment attachment method was from Guam in the 1970s (Birkeland *et al.*, 1979) and this method has advanced each year since. In Okinawa, a total of 100,000 or more fragments were transplanted to approximately 3 hectares of damaged reef over a 6-year period from 2011 to 2016 (Okinawa Prefectural Government, 2017).

The next step to the transplantation is seedling production by sexual reproduction. Coral communities made by transplantation of fragments are likely to have low genetic diversity because most are clones of parental strains. Clones cannot reproduce sexually in most coral species. In addition, generally availability of parent strains is limited. In contrast, we can produce many seedlings (*i.e.* genets) by collecting and fertilizing gametes, rearing larvae, and settling them on substratum.

One of the most efficient methods for gamete collection is through collection of the "slick". In tropical regions, approximately 100 coral species simultaneously spawn gametes on the same night in a "mass spawning" (Harrison *et al.*, 1984). Substantial numbers of fertilized and unfertilized eggs are gathered at the sea surface the following morning; this is called a "coral slick." By collecting these slicks and rearing them, many larvae are obtained at once. Recently, a large-scale slick collection method was developed using a large vessel (Doropoulos *et al.*, 2019). However, it is possible that natural slicks may not form or be detected due to inclement weather conditions. In addition, natural spawning and fertilization may decrease after disturbances because these events reduce the population density of adult corals.

Methods for outplanting are very important because all seedlings (also called fragments) are required to be outplanted on the seafloor. Random outplanting to copy natural coral communities may be manageable on a small scale, similar to gardening. However, for large-scale restoration, there is risk that all the required cost and effort will be wasted by a single disturbance. Therefore, the key to restoration is the concept called "artificial spawning hotspots" (Zayasu and Suzuki, 2019).

Artificial Spawning Hotspots

The basic ideas for sustainable large-scale coral restoration are: 1) creating a high density of mature colonies of a single species to enhance fertilization and collection of gametes, and 2) sustaining larval supply by protecting these high-density populations.

First, selection of the target species is important for establishment of artificial spawning hotspots. Approximately 800 coral species with different reproductive characteristics are known in the world; some are broadcast spawners and some are brooders (Baird *et al.*, 2009). Also, within the broadcast spawners, some are gonochoric and some are hermaphroditic.

The target corals for artificial spawning hotspots are *Acropora* species, a representative genus of hermaphroditic broadcast spawners. *Acropora* is a dominant group in Indo-Pacific reefs and one of the most diverse genera, which forms various habitats with different colony morphologies among species (Wallace, 1999). Within *Acropora*, the branching species are an especially important target, as stated above, in that they contribute to fisheries resources. Because the coral polyps of branching coral distribute less densely than those of tabular and corymbose species (Suzuki unpubl. data), the number of eggs spawned each year is lower, suggesting that their larval supply is also small. It takes longer for these species to recover through natural recruitment

from local extinction due to severe disturbance. Hence, the efficacy of artificial spawning hotspots would be higher for the branching *Acropora* species.

Indeed, the number of *Acropora* recruits is clearly different among reef habitats. From the results of long-term monitoring of *Acropora* recruits around Ishigaki Island, the number of recruits was extraordinarily high in 2014 in the reef slope after the local extinction in 2011 due to a COTS outbreak. The coral cover visually increased from 2017 where the cohort grew to a visible size (Suzuki unpubl. data). However, there was not high recruitment in the lagoon area over a 10-year period and no coral recovery was found (Suzuki unpubl. data). Artificial spawning hotspots should be established in such places where natural recruitment is always low.

In the concept of artificial spawning hotspots, it is necessary to complete seedling production (from gamete collection to larval settlement on artificial substrate) in the sea, which led to the development of the "larval cradle" (Suzuki *et al.*, 2020). Larval rearing techniques using aquaria have already been developed using fertilized eggs collected in the field from adult corals or reared in a tank (Omori *et al.*, 2004); however, there were no methods for completing the entire process in the field. *Acropora* corals release egg-sperm bundles and burst open at the sea surface, then the gametes are fertilized with the gametes released from other colonies (*i.e.* almost no self-fertilization) (Willis *et al.*, 1997). Utilizing this characteristic, a device that catches released bundles with a large net and holds them until they are competent larvae (called the larval cradle) was invented.

A completed version of the larval cradle consisted of a cylinder with a diameter of 1.7-m and a height of 4.25 m made of 30 μm mesh nylon net (**Fig. 1a**), which achieved the collection and rearing of several million coral larvae with more than 90% fertilization and survival rates. The size of the larval cradle was

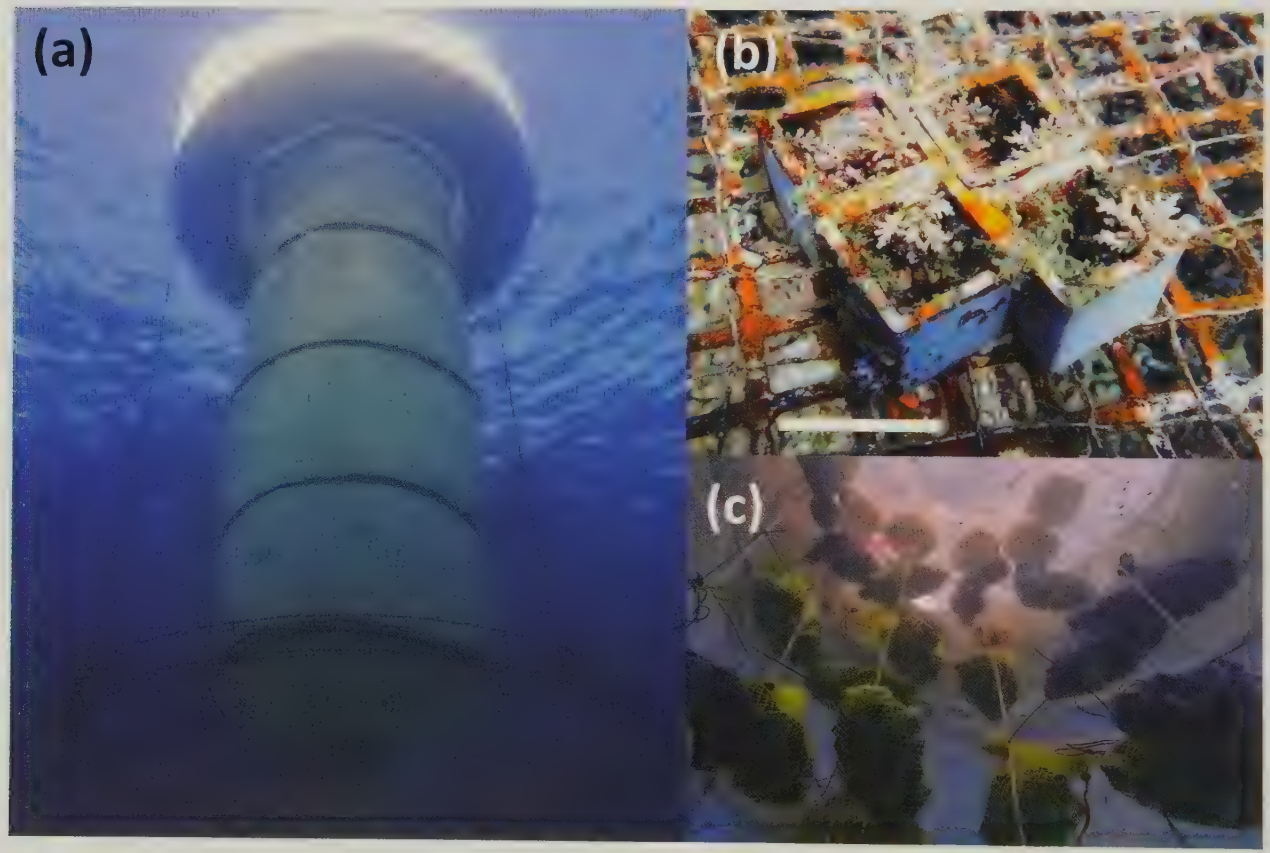

Fig. 1. (a) Setting of the larval cradle with a dedicated float. Size of the cylindrical cradle was 1.7-m diameter and 4-m height below sea level (0.25-m height above sea level). (b) Artificial substrate of short square tube (*i.e.* square hollow section, SHS) for Acropora settlers. Scale bar represents 4 cm. (c) Method of placing the SHSs into the cradle (SHSs were put in net bags and hung from an EVA float).

designed to be easy to handle for a small group of people (*e.g.* fishermen or leisure divers) while ensuring the production of a significant number of larvae. A larger sized larval cradle was not preferred because it would break more easily during stormy weather.

Next, the larvae produced by the larval cradle should be settled on substrates. Post-settlement mortality of coral juveniles is very high. This mortality is primarily caused by unintentional predation by fish grazing on epibenthic fauna and algae (Baria *et al.*, 2010). To prevent fish grazing on coral settlers while keeping moderate light intensity, a lattice-shaped plate was contrived that resulted in good survival of coral juveniles (Suzuki *et al.*, 2011). The short square tube (*i.e.* square hollow section, SHS) substrate was further developed from the lattice-shaped plate (**Fig. 1b**). The SHS substrate was like a single cell of the lattice-shaped plate, which allows us to outplant easily after settled juvenile corals grow up. By hanging the SHSs into the cradle from a buoy (**Fig. 1c**), completion of the settlement process in the sea could be achieved. Settlement of *Acropora* larvae at a moderate density on SHS was also attained by contriving the number of SHSs and the timing and method of placing them into the cradle (Suzuki *et al.*, 2020). The survival rate of the settled corals on SHS was usually 10–20% in the suitable environment (3–15 m in depth, moderate current, little sediment, etc.), although it varied due to the environmental conditions (Suzuki *et al.*, 2013).

Methods for protection of adult corals from disturbances, the second factor for creating artificial spawning hotspots, have also been developed (Suzuki *et al.*, unpubl. data). Bleaching and COTS outbreak are the most serious disturbances for adult *Acropora* corals, and, therefore, the processes of these disturbances were focused for the development of protection methods. One of the available countermeasures against bleaching is shading of corals. It is known that seawater temperature rise of approximately 1℃ causes photoinhibition in the photosynthetic process and generates excess reactive oxygen species that obstruct the symbiosis with zooxantellate algae (Warner *et al.*, 1999). Thus, shading corals during high temperature periods could reduce the mortality by buffering photoinhibition (Coelho *et al.*, 2017). In addition, moving the corals to deeper locations could also reduce light intensity. Another candidate countermeasure against bleaching is reducing starvation of the corals. It is highly probable that the direct cause of death for bleaching corals is starvation by losing nutritional supplementation from symbiotic algae (Borell and Bischof, 2008). Hence, if any nutrition could be artificially supplied during bleaching, mass mortality events may be avoided.

A countermeasure that addresses COTS outbreaks is easier relative to countermeasures that address bleaching. Because COTS cannot climb on thin rods, 50-cm bottom-raised racks with, reinforcing steel rods, is sufficient to prevent COTS damage. In fact, only corals on bottom-raised racks survived the COTS outbreak in 2010–2011 in the Urasoko Bay, Ishigaki Island, where 99% of *Acropora* corals within the bay were eaten by COTS (Suzuki unpubl. data).

By using these countermeasures for corals in artificial spawning hotspots, the larval supply from the hotspots could be several hundred times higher than that of a wild *Acropora* population experiencing the same environmental conditions (**Fig. 2**). Calculation of the total larval supply in a longer span showed an even larger gap between the hotspot and wild population. That is, the larval supply from the hotspots could be stable annually for a long period, while the gradual deterioration of wild populations is predicted over 50–100 years (IPCC, 2018).

There may be concern with species diversity of target corals for restoration. However, only 5–10 branching *Acropora* species dominate the lagoon area in Indo-Pacific reefs, covering more than 50% of the total coral area, even though more than 100 coral species were recorded in the area. In other words, the larval supply from the artificial spawning hotspots of 5–10 *Acropora* species is comparable to that from the wild coral community in terms of species diversity. In addition, the phase shift from corals to macroalgae in a damaged reef is considered to delay the recovering of coral communities after disturbances (Kuffner *et al.*, 2006; Bozec *et al.*, 2019). Rapid restoration of dominant species could be effective to avoid or reverse such a phase shift to macroalgae.

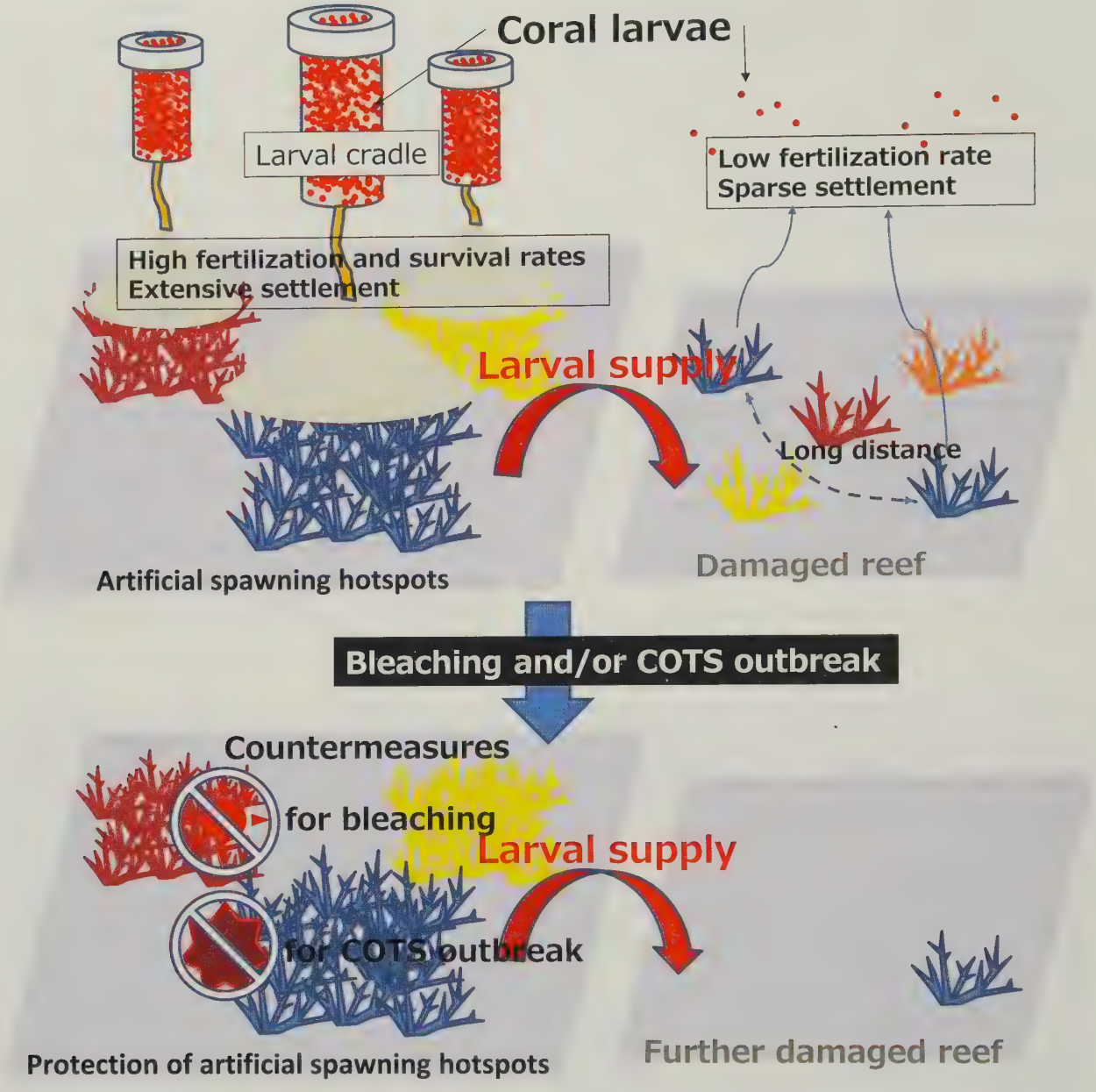

Fig. 2. Image of artificial spawning hotspots concept. Different colors represent different species of *Acropora* corals.

Direct Larval Seeding

Direct seeding of mass larvae acquired from larval cradles or natural slicks would be a shortcut to large-scale coral restoration (Heyward *et al.*, 2002). However, at present, direct larval seeding on the seafloor remains impractical due to low settlement and survival rates of the seeded larvae (*e.g.* Edwards *et al.*, 2015). In the Philippines, approximately 0.4 million larvae were directly seeded to several tens of m^2 of damaged reef and 2.3 colonies per m^2 was found 3 years after the seeding (dela Cruz and Harrison, 2017). An enclosing net was used to prevent dispersal of the larval seeding in that study; however, it is difficult to apply this method on a larger scale (*e.g.* more than 1 hectare). Ultimately, the direct seeding of an overwhelming number of larvae with high density that is comparable to natural recruitment would be effective in large scale restoration even if the larvae were seeded without an enclosing net. Studies to determine suitable seeding methods, including the necessary minimum

density, are currently in progress. In addition, there are some places where larval seeding has no effect. In the sandy bottom, covering most of the lagoon, few settlement substrata such as rock reefs are available. It is expected that outplanting or seeding of corals settled on the artificial plates is more effective in such places (Chamberland *et al.*, 2017).

In conclusion, the methods and techniques reviewed in this paper can be effectively used for gamete collection, larval rearing, seedling production, direct larval seeding, maintenance of adult corals for larval supply, and protection of adult corals from disturbances in the field (*i.e.* without land facilities). Using these simple methods together shows promise to successfully achieve large-scale coral restoration.

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Annotated Bibliography of Key Works

(1) Zayasu Y., and Suzuki G., 2019: Comparisons of population density and genetic diversity in artificial and wild populations of an arborescent coral, *Acropora yongei*: implications for the efficacy of "artificial spawning hotspots". *Restor. Ecol.*, **27**, 440-446.

The authors assessed population density and genetic diversity of a wild, arborescent coral, *Acropora yongei*, and compared these parameters with those of an artificially established *A. yongei* population in the field. The population density of wild arborescent corals was only 0.27% of that in the artificial population, even in a high-coverage area. Genetic diversity was also low in the wild population compared with the artificial population, and approximately 10% of all wild colonies were clones. Based on these results, the larval supply in the artificial population was estimated to be at least 1,400 times higher than that in wild *A. yongei* populations for the same area of adult population.

(2) Suzuki G., Yamashita H., Kai S., Hayashibara T., Suzuki K., Iehisa Y., Okada W., Ando W., and Komori T., 2013: Early uptake of specific symbionts enhances the post-settlement survival of *Acropora* corals. *Mar. Ecol. Prog. Ser.*, **494**, 149-158.

The authors tested the hypothesis that early acquisition of symbionts enhances post-settlement survival. Symbiotic and aposymbiotic *Acropora* larvae were prepared in the laboratory and settled

on experimental plates in the field. The survival of settlers was monitored for 15 months, and the results showed that more larval-stage settlers harbouring symbionts survived than those without. The higher survival rate of 'early uptake' corals was more pronounced on shaded plates. These results suggest that the early uptake of specific symbionts enhances post-settlement survival in dark places such as reef crevices, which are sites commonly settled by coral larvae.

(3) Suzuki G., Kai S., Yamashita H., Suzuki K., Iehisa Y., and Hayashibara T., 2011: Narrower grid structure of artificial reef enhances initial survival of in situ settled coral. *Mar. Pollut. Bul.*, **62**, 2803–2812.

The authors demonstrated through field experiments that the design of artificial grid plates may influence the initial survival of *Acropora* corals, with narrower grids being the most effective. In fact, grid plates with a 2.5-cm mesh presented the highest recorded survival rate (14%) at 6 months after settlement (representing approximately 50 corals per 0.25 m^2 of plate). This was the first study where such high survival rates, matching those of cultures under aquarium conditions, were obtained in the field without using additional protective measures, such as guard nets against fish grazing after seeding. Therefore, their results provide a foundation for establishing new and effective coral restoration techniques for larval seeding.

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The influence of climate and environment on the growth and survival of Pacific oyster seed in US West Coast estuaries

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Abstract: Pacific oysters *Crassostrea gigas* were introduced to the US west coast in the early 1900's and were initially raised directly from seed (juveniles that had set naturally on cultch shell) imported from Japan. Oysters regularly spawned and became "naturalized" in only several selected West coast locations, such as Willapa Bay, Washington, where conditions allowed for both adult oyster spawning and larval survival, retention and settlement. The shellfish industry relied on "natural or wild" caught seed from these locations or continued seed imports until the advent of hatchery technology in the late 1970's. Recent larval mortality events in hatcheries have been linked directly to changes in seawater chemistry with high pCO_2 conditions and acidified water associated with seasonal upwelling along the U.S. West Coast. These conditions may have also resulted in reduced natural oyster sets in Willapa Bay, but estuarine gradients in water chemistry and temperature add complexity making this more difficult to discern. Shellfish hatcheries have adapted to these conditions by measuring seawater carbonate chemistry, buffering incoming water, and adjusting the timing of larval production cycles. While there appear to be larval fitness traits that are genotype-dependent, the potential for breeding programs to improve OA specific traits is only currently receiving attention and remains uncertain. Experiments have rarely been conducted that distinguish success at important physiological transitions in the larval life history and potentially also at the juvenile seed stage when these oysters are out-planted to estuaries with variable conditions.

We review results from recent experiments where survival of oyster larvae raised from crosses of "wild" parental broodstock collected in Willapa Bay was compared to that of larvae raised from controlled crosses with improved lines created by the Molluscan Broodstock Program (MBP) at the Hatfield Marine Science Center (HMSC), Oregon State University. The MBP breeding program was designed to enhance valuable field traits including growth and survival of juvenile and adult oysters, but to date has not explicitly addressed larval traits. Nonetheless, MBP larvae produced more than twice the number of settled spat compared with wild larvae under commercial hatchery conditions (with buffered seawater, pH ~8.3 and Ω_{arag} >2). This advantage also occurred, but to a lesser degree, under unfavorable high pCO_2) conditions (pH ~7 and Ω_{arag} <1). Separate experiments were conducted to evaluate survival of juvenile seed along the estuarine gradient and inside and outside eelgrass (*Zostera marina*), a marine plant which has the ability to modify local seawater chemistry. Growth and survival of juvenile oyster seed varied along the estuarine gradient with fastest growth, but lower survival occurring near the estuary mouth and there was no apparent effect of seed source. Results from experiments where MBP seed was planted both inside and outside eelgrass along these same gradients, suggest that this plant can reduce seed growth, especially at locations away from the estuary mouth.

Keywords: Pacific oyster, juvenile, growth, estuary, climate

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Introduction

The Pacific oyster *Crassostrea gigas* was introduced to estuaries along the US West Coast in the early 1900's and largely replaced the native oyster *Ostrea lurida*, which had been extensively fished commercially and mostly overharvested or succumbed to other factors including pollution (Blake and Ermgassen, 2015; Dumbauld *et al.*, 2011; Polson and Zacherl, 2009; Steele, 1964). Pacific oysters were cultured and harvested from leased and privately owned estuarine tidelands and the industry either relied on juvenile seed oysters shipped from Japan or placed cultch shell in several areas where this oyster had naturalized and conditions were conducive for them to spawn (*e.g.* Dabob and Willapa Bay in Washington State, USA and/or Ladysmith Harbor and Pendrell Sound in British Columbia, Canada) (Quayle, 1988). The advent of local hatchery production of oyster larvae and oyster seed in the late 1970's closed the culture life cycle separating the two life history phases and changed the nature of the industry again (Chew, 1984).

The nearshore coastal ocean along the US west coast is part of the California current ecosystem, an eastern boundary upwelling system. Oysters cultured in these systems are thus subject to seasonal events where cold, nutrient rich, high pCO_2 water is transported to the surface and episodic intrusions into estuaries can significantly lower pH and the aragonite saturation state especially near the estuary mouth (Feely *et al.*, 2010; Hauri *et al.*, 2013). Shellfish hatcheries operating in this region have experienced significantly reduced rates of larval growth and survival during these upwelling events (Barton *et al.*, 2012). In response to these challenges, commercial hatcheries now measure seawater carbonate chemistry, strategically time larval production cycles, and chemically buffer incoming seawater in order to maintain optimal carbonate chemistry conditions (Barton *et al.*, 2015).

While there are important exceptions like the Salish Sea in Washington State, most U.S. West coast estuaries are small relative to the spatial extent of the nearby open coastline, so these upwelling events and changes in seawater chemistry can also result in larval oyster mortality and reduced or failed oyster seed sets in estuaries where naturalized oyster populations reproduce. Willapa Bay, which is one of the single largest cultured oyster production sites in the US and the third largest estuary on the US west coast has only a 358 km^2 signature, which roughly equals the size of the James River sub-estuary in Chesapeake Bay, the largest estuary on the US East Coast (11,600 km^2). Estuaries like Willapa Bay also experience less riverine influence during the summer months and have shorter residence times than estuaries where oysters are present on eastern edges of continents (Hickey and Banas, 2003). Gradients in water chemistry and temperature are greatly influenced by short term events that not only influence larvae, but also timing and magnitude of spawning events and retention of larvae in these estuaries making it more difficult to discern reasons for failures in larval seed set (Hales *et al.*, 2017; Ruesink *et al.*, 2018).

Improved larval survival and growth under high small 10 day post metamorphosed oyster spat were CO_2 conditions appear to be genotype-dependent fitness traits (Frieder *et al.*, 2017; Pan *et al.*, 2018; Sunday *et al.*, 2011), but the potential for breeding programs to improve these traits has not yet received much attention. A collaborative effort between scientists at Oregon State University and the US West Coast shellfish industry known as the Molluscan Broodstock Program (MBP) was initiated in 1996 to enhance juvenile oyster survival and growth (de Melo *et al.*, 2016; de Melo *et al.*, 2018; Langdon *et al.*, 2003). This selective breeding program uses family-based mating designs and bi-parental crosses to maintain genetic diversity and limit effects of inbreeding. Although seven generations of larval cohorts have been reared in the hatchery, the program is targeted to improve yield of juveniles and adults on farms and there has been no direct attempt to select for larval performance traits. Nonetheless, commercial shellfish hatcheries that partner with MBP reported that larvae spawned from MBP broodstock survived and grew better when compared to wild counterparts, especially during periods of strong upwelling, so experiments were conducted to discern whether unintentional selection for this trait had occurred. Results demonstrated that larvae from MBP

broodstock produced from 37% to 50% more spat than larvae from "wild" broodstock collected from a naturalized population in Willapa Bay (Durland *et al.*, 2019). This advantage occurred under both commercial hatchery conditions (with buffered seawater, pH ~8.3 and Ω_{arag} >2) and to a lesser degree, under unfavorable high pCO_2 conditions (pH ~7 and Ω_{arag} <1). This suggests that improvements in larval performance had occurred despite no intentional selection. Further analysis of single nucleotide polymorphisms (SNPs) from pooled larval DNA indicated that larvae from wild stocks had more than twice the number of loci affected by acidified culture conditions, compared to larvae from MBP stocks (Durland, 2019). Functional analysis revealed that the predicted genes associated with changes under OA conditions were linked to the structure and function of cellular membranes (De Wit *et al.*, 2018), but the affected loci were largely exclusive to each parental stock with little overlap, suggesting that development of universal markers and selecting for OA resistance in Pacific oysters will be complicated.

The effect of altered water chemistry on juvenile oysters once they settle naturally or are transplanted from hatcheries and planted on farms for grow-out are less studied. Delayed carry-over effects on juvenile growth due to larval exposure (Gobler and Talmage, 2013; Hettinger *et al.*, 2013) and cross-generational effects when breeding adults are exposed to elevated CO_2 have been documented (Parker *et al.*, 2015; Parker *et al.*, 2012), but distinguishing the effects of elevated CO_2 and reduced aragonite saturation from other environmental factors in estuaries is difficult. Hollarsmith *et al.* (2020) documented the influence of seasonal processes on both water chemistry and temperature along the estuarine gradient in Tomales Bay, CA. They found that both juvenile Pacific and native oyster growth and survival was more influenced by freshwater runoff during the wet season (though limited in this estuary) than by carbonate chemistry during the summer upwelling season. Growth was highest during the upwelling season and appeared to be related to higher phytoplankton concentrations in the water column especially near mid-bay where other stressors (low dissolved oxygen, low salinity, high temperature) were less evident.

Finally, there continues to be interest in evaluating the potential role of seagrasses as refugia from the effects of altered water carbonate chemistry in part because these important estuarine plants may also grow more rapidly under elevated CO_2 and increase carbon sequestration (Washington State Blue Ribbon Panel on Ocean Acidification, 2012; Cullen-Unsworth and Unsworth, 2016; Kelly *et al.*, 2011). Seagrasses uptake CO_2 while photosynthesizing during the day and therefore have the potential to modulate carbonate chemistry at least at very local scales, but they also respire at night and die back during the winter in most temperate estuaries potentially increasing pCO_2 (Hendriks *et al.*, 2014). A laboratory evaluation of the interaction between juvenile oysters, the seagrass *Zostera marina* (hereafter eelgrass) and seawater carbon chemistry showed that enhanced daytime seawater pH in the presence of eelgrass did not counteract the negative effects of increased pCO_2 when both oysters and eelgrass were present (Groner *et al.*, 2018). However studies conducted in estuaries suggest that growth and survival of juvenile oysters can be enhanced in eelgrass (Lowe *et al.*, 2019; Smith, 2016). This effect was site specific and therefore difficult to attribute to water chemistry and distinguish from effects of other factors like reduced water flow in eelgrass which potentially results in less suspended sediment in the water and enhanced phytoplankton food intake.

Here we present preliminary results from two experiments where juvenile oyster seed was planted inside and outside eelgrass at several locations along the axis of two US West coast estuaries where we expected gradients in water chemistry and environmental conditions to differ.

Methods

We conducted experiments in two estuaries along the US West Coast. Netarts Bay, Oregon, USA (45°25' N, 123°56' W) is a small tidally flushed estuary, with over ~75% of its total volume turning over every tidal cycle (6.22 hours) (**Fig. 1**). It has a relatively small (36.3 km) watershed whose summertime

freshwater contribution to flushing is negligible (Glanzmann *et al.*, 1971; Kentula and McIntire, 1986). Patterns in carbonate chemistry were therefore expected to be almost entirely driven by conditions on the coastal shelf, perhaps at both the marine end near the estuary mouth and at the riverine end during the summer when this experiment was conducted. Netarts Bay is also home to the Whiskey Creek Shellfish Hatchery, the largest independent producer of oyster seed in the Pacific Northwest (Barton *et al.*, 2012) where a continuously-monitoring carbonate chemistry analyzer has been in place since 2010 (Barton *et al.*, 2015). Though still dwarfed in size by estuaries along the US East coast as noted above, Willapa Bay, Washington USA (46°32' N, 123° 59' W) is the third largest estuary along this coast. It has a much larger watershed than Netarts Bay (2,857 km^2) (**Fig. 2**), but is still strongly tidally-influenced, especially in the summer when river flow is virtually non-existent. Though roughly half the water volume

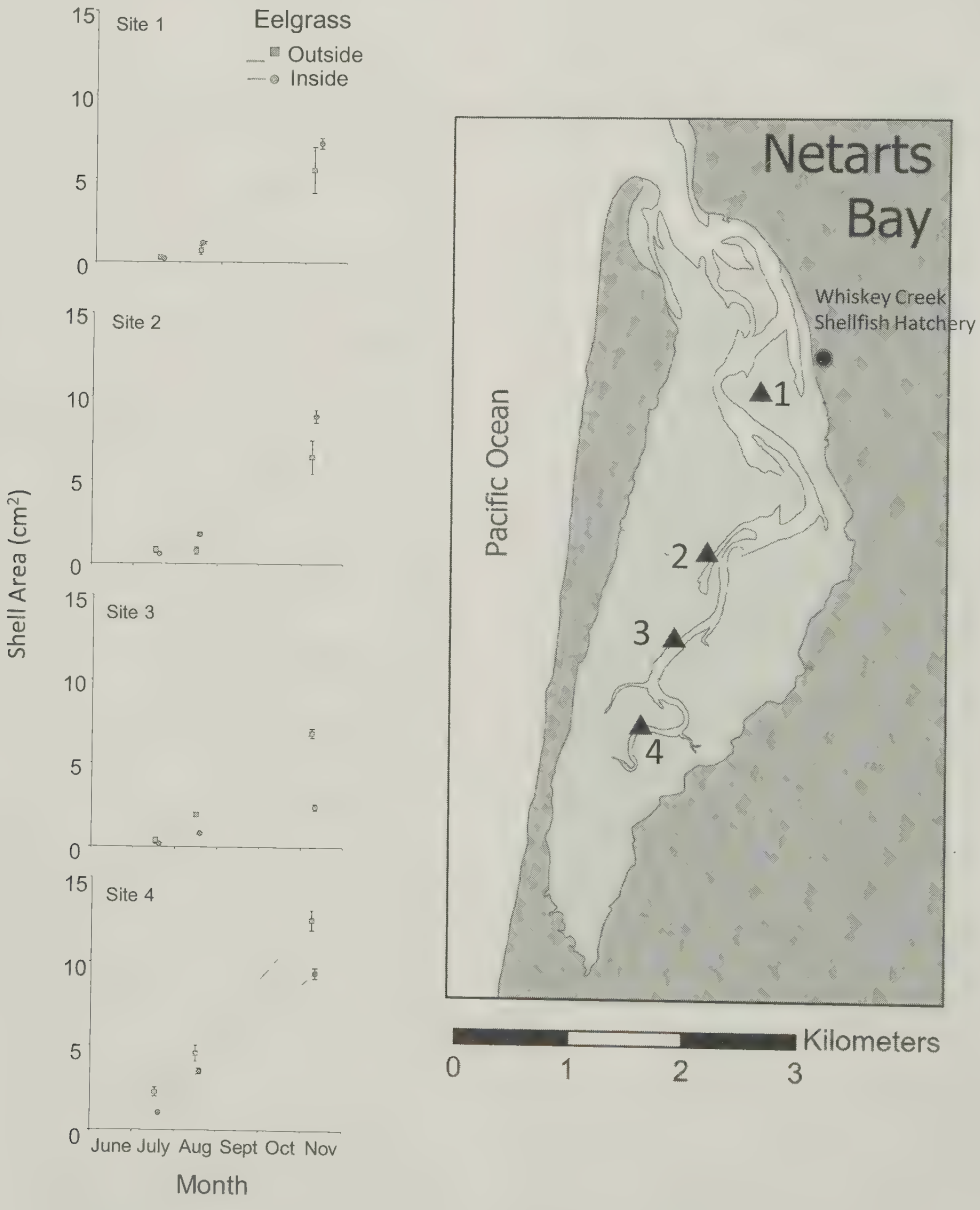

Fig. 1. Results of the 2015 experiment in Netarts Bay, Oregon, USA. Oysters were deployed in May at 4 sites shown in map (right). Average shell area measurements of oysters ($cm^2 \pm SE$) in eelgrass habitat (green) and outside open habitat (orange) is shown for three time points in July August and November (left).

is exchanged on every tide and replaced with new ocean water, the water at the southern end of the estuary has a 3 to 5 week residence period (Banas *et al.*, 2004; Banas *et al.*, 2007). It is the single largest producer of oysters on the US West coast and often in the US. Carbonate chemistry has been shown to be influenced by upwelling near the ocean endpoint at the estuary mouth, but also by alkalinity and other factors near the Naselle River (Ruesink *et al.*, 2018; Ruesink *et al.*, 2015), so we expected a potentially different gradient and influence of carbonate water chemistry on oyster survival and growth in this estuary than Netarts Bay.

Experimental plantings of juvenile oyster seed (spat) were made in two separate years to contrast growing conditions inside and outside of eelgrass at

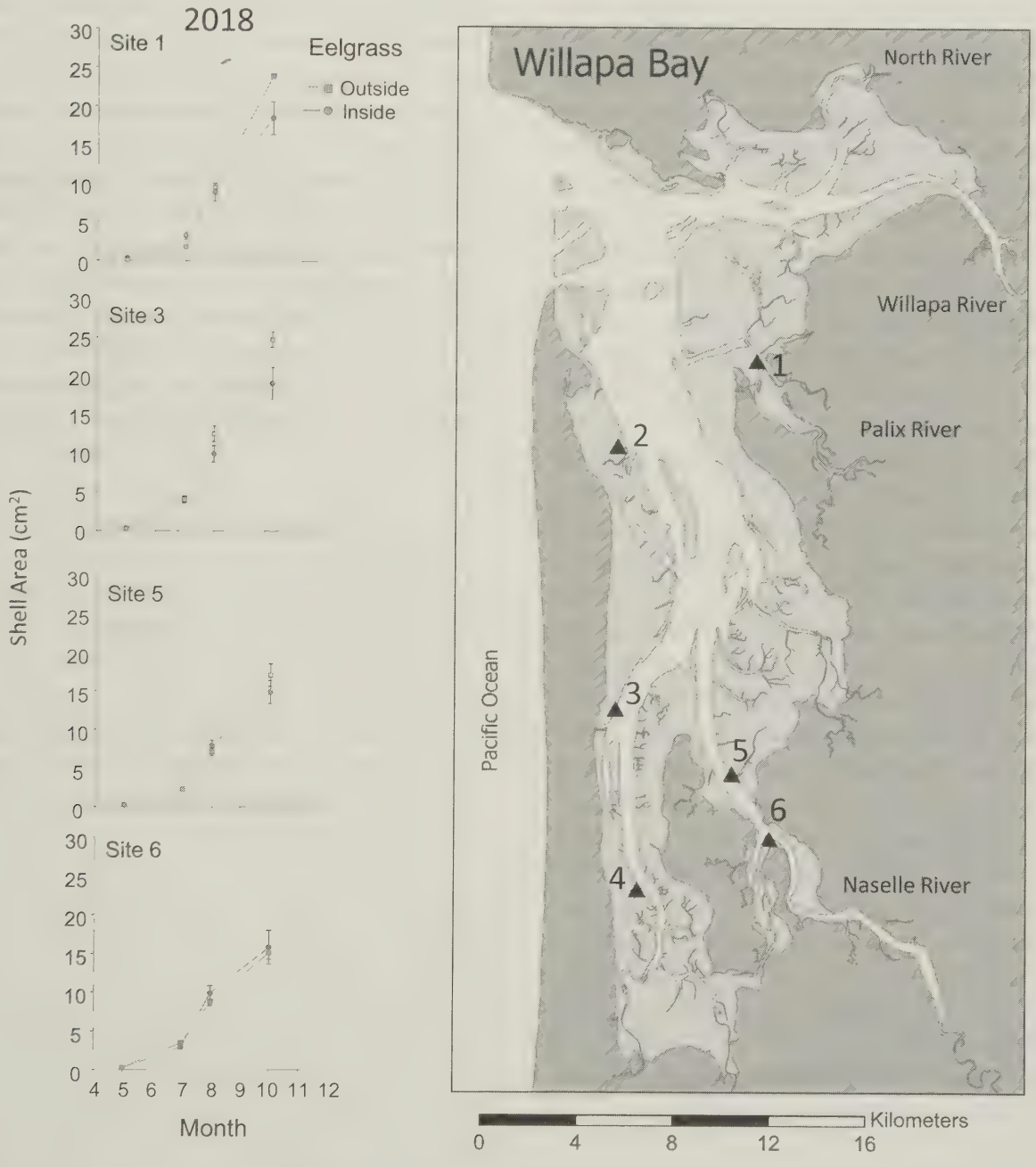

Fig. 2. Results of the 2018 experiment in Willapa Bay, Washington, USA. Oysters were deployed in May at six sites (map on right). Average shell area measurements of oysters ($cm^2 \pm SE$) in eelgrass habitat (green) and outside open habitat (orange) is shown for three time points in July August and October.

several sites along the estuarine gradient in these two estuaries. Our methods differed slightly and were briefly as follows:

Netarts Bay 2015

Aged Pacific oyster shell was used as cultch for this experiment. Five shells were labeled with an individual plastic identification tag and placed in each of 32 small mesh bags (2.5 cm mesh) and these placed in a setting tank at the Whiskey Creek Shellfish hatchery on 5/11/2015. Eyed oyster larvae (MBP Pod, cohort 26) were placed in the tank and allowed to set on these shells following standard hatchery practices. Bags were removed from the tanks and deployed at 4 sites along the estuarine gradient in Netarts Bay (**Fig. 1**) on 5/20/2015. Four shell bags were deployed within eelgrass and four outside eelgrass in bare habitat at the same tidal elevation. Shell bags were attached to PVC pipes so they were suspended approximately 20 cm above the sediment. Poles with bags were placed approximately 5 m apart and each pole had copper tape wrapped around it at the sediment surface in order to prevent access by oyster drill predators. Oyster shells were removed from the bags and photographed at roughly monthly intervals thereafter and returned to the lab for final measurements on 9/30/2015. Temperature was recorded continuously at 15 min intervals using Hobo® data loggers. Photographs from each time point were examined and oyster measurements (length and width) made using Image J (v1.49®) on size calibrated images. Oyster cultch shell labels and shape enabled each individual oyster to be labeled and recorded so that they could be identified and counted in subsequent photos. An assessment of fouling which became an obvious potential factor (% cover, mostly barnacles) was also made. At the conclusion of the experiment oysters were counted, measured (length, width in cm) with calipers, removed from the cultch shell and both dry and wet meat and shell weight measurements made.

Willapa Bay 2018

After being set as singles in the MBP hatchery at HMSC, five oyster spat were cemented (cyanoacrylate glue) to the rough side of small ceramic tiles (5cm × 5cm) in this experiment. Tiles were labeled with an individual plastic identification tag and then held for 24 hours in tanks before being transported and deployed at 6 sites along the estuarine gradient in Willapa Bay, Wa (**Fig. 2**) on 5/20/2018. Tiles were attached to PVC stakes at two positions: 20 cm above the sediment and just above the sediment surface. Two tiles were attached back to back at each position: one with spat from an MBP cohort and one with spat from broodstock collected from a "wild" population in Willapa Bay. Three stakes with tiles were deployed within eelgrass and three outside eelgrass in bare habitat at the same tidal elevation at each site. Poles with tiles were placed approximately 10 m apart and each pole had copper tape wrapped around it at the sediment surface in order to prevent access by oyster drills. Tiles were visited and photographed at roughly monthly intervals thereafter and returned to the lab for final measurements on 10/30/2018. Fouling organisms were removed from areas surrounding each oyster using a toothbrush before photographs were taken at each time point. Photographs from each time point were examined and oyster measurements (length and width) made using Image J (v1.49®). Temperature was recorded continuously at 15 min intervals using Hobo® data loggers deployed in two habitat treatments at each site and YSI® sondes were deployed at two locations (Nahcotta and Palix) for 24 hours in August. At the conclusion of the experiment oysters were counted, measured (length and width in cm) with calipers, scraped from the tile and both dry and wet meat and shell weight measured.

Data Analysis

Data from both experiments were analyzed using general linear and linear mixed effect models in R (R Core Team, 2019). Here we present data for shell size (length × width) examined over the summer deployment period. Because we were able to track individual oysters in both experiments, we then evaluated oyster growth for discrete time intervals for which we had the most complete data: 1) an initial interval fairly close to deployment when oysters first experienced estuarine conditions and 2) over the entire period or at the end of the period

when we collected the oysters and made additional measurements (*e.g.* tissue and shell mass). For the 2015 Netarts Bay experiment there were two categorical factors of interest represented in this model: eelgrass (present or absent) and location (4 sites). We also examined and added the proportion of each cultch shell covered with fouling organisms (mostly barnacles) as a factor in this model for the initial period where we collected this information. Models for the second 2018 Willapa Bay experiment included the same primary categorical treatments: eelgrass (present or absent) and location (6 sites), but also included height (20cm above sediment, on bottom) and seed source (MBP, Wild). Growth was evaluated as a normalized daily average = final size (area in mm^2) - initial size (area in mm^2) / days in interval / initial size. Complete models with interaction terms were initially examined, the most important random component determined if needed, and then the most parsimonius model selected by comparing full and nested models with log-likelihood tests and comparing Akaike information criterion (AIC).

Results and Discussion

Oyster growth differed along the estuarine gradient with oysters reaching a larger size outside eelgrass than within eelgrass at the two southernmost locations in Netarts Bay in 2015 and reaching the largest size at site 4 located furthest from the estuary mouth (**Fig. 1**). The opposite pattern was observed at the two sites closer to the estuary entrance, where oysters grew to a larger size within eelgrass resulting in significant interaction between location and eelgrass factors in models for final shell size, but not for normalized growth from August to November (**Table 1**). Oyster mortality across the whole season was highest at locations away from the ocean, but there was no discernable trend attributable to eelgrass presence. Fouling on the cultch shells, mostly by barnacles, was apparent at the first sampling effort in July, highest at sites closest to mouth and higher outside eelgrass than inside eelgrass (**Fig. 3**). Fouling was not a significant factor however when included in the models for size or growth at this time point (**Table 1**). Smith (2016) conducted experiments at site 1 in Netarts Bay during the same year, but repeatedly deployed small 10 day post metamorphosed oyster spat monthly from March - October and monitored for short term growth and mortality. Smith (2016) documented a significant effect of eelgrass on in- situ carbonate chemistry, but only observed significant growth and survival advantages for oyster spat placed in eelgrass during May. Survival of these

Table 1. Results of linear mixed effect models ("nlme" package, Pinheiro *et al.* 2019) used to examine the effect of habitat and site on shell area and growth of oysters in Netarts Bay in 2015. Individual cultch shells were used as random intercept terms in each model

		Full Model			Final Model		
Response	factor	df	F	*p*	df	F	*p*
Shell area (Nov)	site	3,92	27.41	<0.001			
	eelgrass	1,92	19.56	<0.001			
	site*eel	1,92	11.30	<0.001			
Normalized growth (Aug - Nov)	site	3,94	14.50	<0.001	3,94	13.34	<0.001
	eelgrass	1,94	9.40	0.003	1,94	9.0	0.004
	site*eel	1,94	0.26	0.856			
Shell area (July)	site	3,84	43.45	<0.001	3,89	42.31	<0.001
	eelgrass	1,84	14.64	0.002	1,89	3.76	<0.001
	barnacles	1,84	3.68	0.058			
	site*eel	3,84	0.41	0.744			
	barn*eel	1,84	0.31	0.576			

small spat planted in subsequent months appeared to correlate with an overall declining trend in daily pCO_2 minima. These findings agree with other research and suggest that additional variables like water flow and tidal exposure must be taken into account (Koweek *et al.*, 2018).

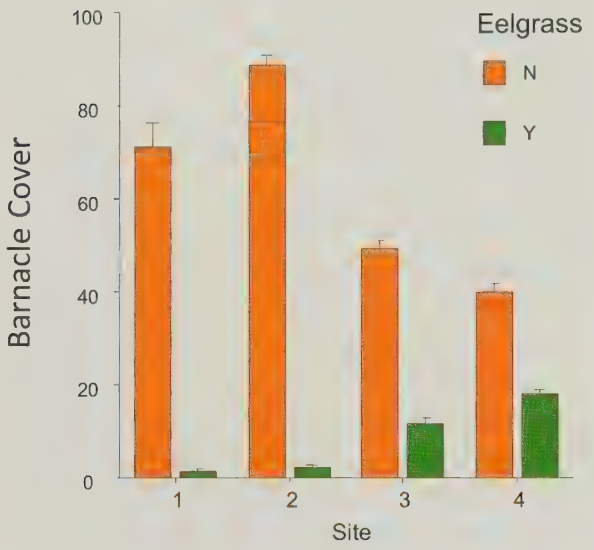

Fig. 3. Comparison of average percent barnacle cover measured on shells deployed in eelgrass (green bars) and outside eelgrass (brown bars) at each site in Netarts Bay during July 2015.

Though experimental design and deployments were slightly different than those in the 2015 Netarts experiment, oyster growth also differed along the estuarine gradient in Willapa Bay in 2018. Oysters consistently reached a larger size at locations closest to the estuary mouth in this estuary (Sites 1–3) (**Fig. 2**), but few oysters survived at Sites 1 and 2 due at least in part to experimental problems (early losses of glued oysters from tiles and lost stakes). Oysters obtained a larger size outside of eelgrass than within eelgrass at these locations, but there was significant interaction between location and eelgrass in models for both shell area and normalized growth (**Table 2**), because no eelgrass effect was observed at locations away from the mouth (Sites 4–6) (**Fig. 2**). We also deployed oysters from two parental sources (selected MBP family and "wild") at two heights above the sediment in this experiment. While there was no significant effect of oyster source, there was significant interaction between height above sediment and location (**Table 2**). Oysters grew larger 20 cm above the bottom at locations near the estuary mouth and the opposite effect was observed at sites 5 and 6. Previous authors also found enhanced

Table 2. Results of general linear models used to examine the effect of habitat (eelgrass, open), seed source (MBP, Wild), position (off bottom, on bottom), and site on shell area and growth of oysters in Willapa Bay in 2018. Only sites 3–6 used in these models due to lack of sufficient data at sites 1–2

		Full Model			Final Model		
Response	factor	df	F	*p*	df	F	*p*
Shell area (August)	site	3	9.31	<0.001	3	9.47	<0.001
	eelgrass	1	3.59	0.061	1	3.66	0.058
	position	1	2.48	0.118	1	2.52	0.115
	source	1	3.57	0.061	1	3.63	0.059
	site*eel	3	3.97	0.010	3	4.04	0.009
	site*pos	3	3.41	0.020	3	3.47	0.018
	site*source	3	0.35	0.789			
Normalized growth (July – Aug)	eelgrass	1	0.001	0.970	1	0.001	0.970
	site	3	3.19	0.026	3	3.15	0.026
	position	1	0.49	0.487	1	0.49	0.487
	source	1	1.24	0.267			
	site*eel	3	2.45	0.067	3	2.53	0.060
	site*pos	3	3.02	0.033	3	3.02	0.044
	site*source	3	1.25	0.295			

growth of Pacific oysters near or close to the mouth of this estuary (Ruesink *et al.*, 2003). Lowe *et al.* (2019) also documented little difference in oyster growth between eelgrass and open habitat treatments at the same riverine endpoint (site 6). They found significantly larger oysters in eelgrass than outside this habitat at their site closest to the ocean. While we observed the opposite trend, this agrees with our observations for Netarts Bay above. These authors and others (Hollarsmith *et al.*, 2020) mostly attribute trends in oyster growth to the concentration of phytoplankton as food and found that trends in survival might also reflect predator abundance or even abundance of bacteria and disease as stressors in these two habitats rather than water chemistry. In single 24 hour records, we observed higher levels of food (measured as relative chlorophyll a) within eelgrass at two of the stations along this gradient in Willapa Bay and more dramatic fluctuation at the location closest to the estuary mouth where we also observed colder average temperatures over the entire experimental period and greater fluctuations and differences in pH (**Fig. 4**). This location (site 1) is however potentially also more affected by freshwater

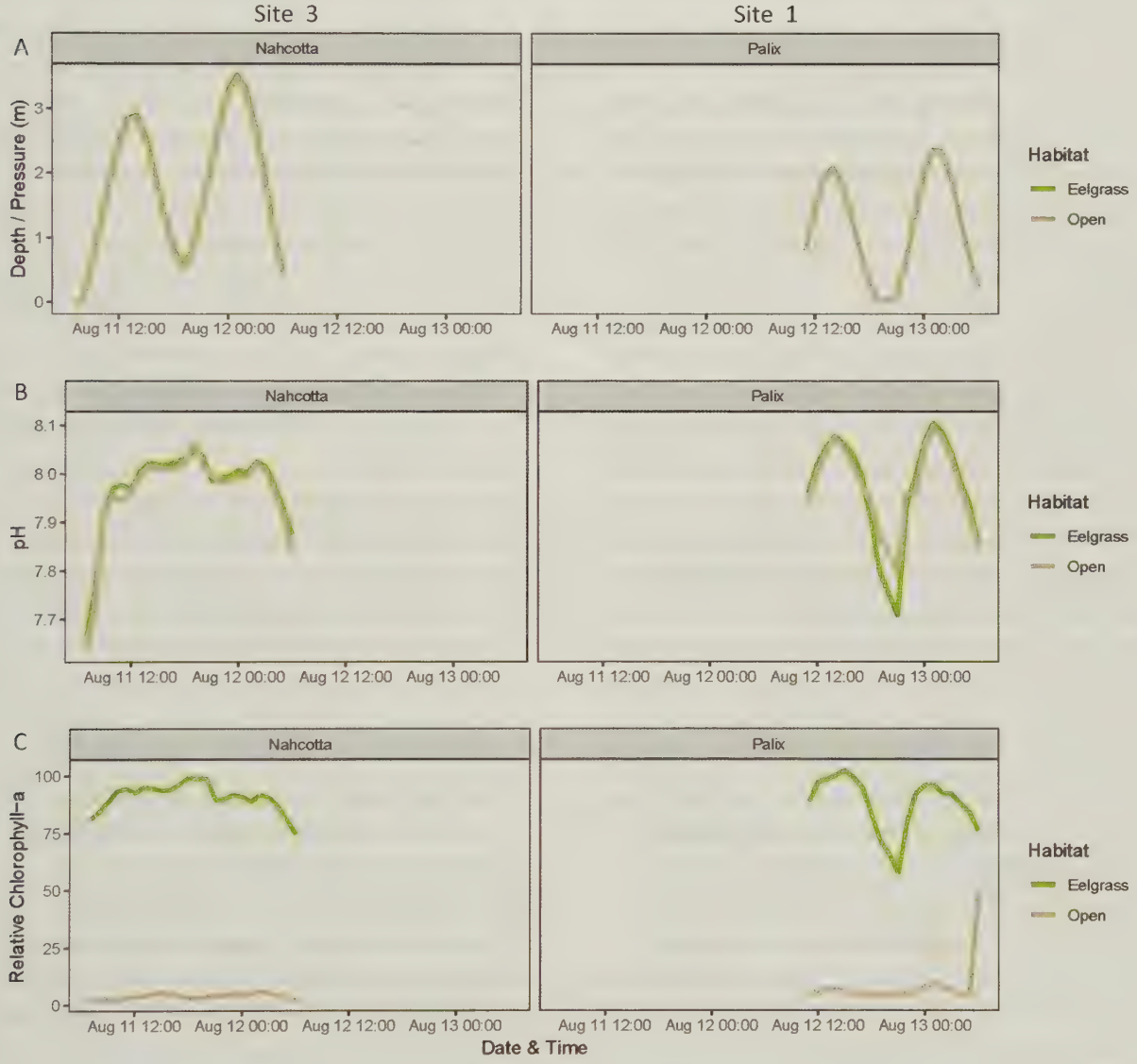

Fig. 4. Twenty-four hour records for A) water depth – following the tide, B) pH , and c) relative chlorophyll a, at intertidal sites 1 (Palix River) and 3 (Nahcotta) where oysters were deployed in Willapa Bay. Note the fluctuating values of both parameters at site 1, but differences in pH in eelgrass at low water at night at this site and consistently higher chlorophyll a in eelgrass at both locations.

from the Palix River as potentially evidenced by a drop in pH and chlorophyll a at low tide. We have not yet directly related this environmental data directly to oyster growth and survival, in part because we recognize that the density and spatial configuration of eelgrass will also affect water flow and food supply so static measurements and simple site/treatment characterizations and point in time estimates of these water chemistry parameters need to be refined.

Our preliminary conclusions are:

- Oyster larvae have clearly been shown to be sensitive to water chemistry including low aragonite saturation state, but other conditions are important and genotype/broodstock may play an important role in initial spat settlement and size.
- Location or planting site for juvenile spat (seed) within the estuary is important. Juvenile oyster seed generally grows faster near the mouth of US West Coast estuaries, but there are important finer scale, within estuary spatial and temporal gradients.
- The effect of eelgrass on oyster seed also depends on planting site. We suspect this is due to several interacting factors including water flow, food supply, and perhaps settlement and competition with other invertebrates, and not water temperature or modifications to water chemistry. However, this data and relationship remains to be collected and further examined at appropriate spatial and temporal resolution.
- Juvenile oyster growth is generally faster above the sediment surface, but this varies along the estuarine gradient as well. This is also likely due to food supply and perhaps other factors influencing oyster feeding behavior including sediment load but needs to be further explored in these estuaries.

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This is a review of the history and science underpinning the effects of changing seawater chemistry on bivalve shellfish larvae and the impacts that have already taken place to the commercial shellfish aquaculture industry on the US West Coast. Multiple authors contributed to this review which addresses a broad audience but covers the leading research on direct effects to bivalve larvae as well as monitoring seawater conditions and adapting to these changes.

(2) Durland E., Waldbusser G., and Langdon C., 2019: Comparison of larval development in domesticated and naturalized stocks of the Pacific oyster *Crassostrea gigas* exposed to high pCO_2 conditions. *Mar. Ecol. Prog. Ser.*, **621**, 107-125.

Two replicated experiments were conducted where Pacific oyster larvae from "wild" parent crosses and those from selected stocks (OSU Molluscan Broodstock Program) were raised and set under favorable hatchery conditions (buffered seawater, pH ~7.8 and Ω_{arag} ~2) and unfavorable (pH ~7.4 and Ω_{arag} ~1) conditions. Early larval development was inhibited by acidified seawater and it affected the timing, but not magnitude of larval mortality. The effect on metamorphosis (setting) was variable, but MBP larvae produced more and larger spat in ambient and high pCO_2 seawater, respectively.

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The authors present detailed data on seawater chemistry (especially pCO_2 and aragonite saturation state) for Willapa Bay, Washington where Pacific oysters have been the mainstay of the oyster aquaculture industry for almost 100 years and there is a long term record of spawning and setting. They reconstruct this record for a longer historical period and their data suggest that recent conditions provide a smaller window of optimal conditions (low aragonite saturation state and warm enough temperatures for oyster spawning) than occurred historically. While they did not sample larvae and therefore can't confirm effects, they substantiate the complexity of measuring these effects and attributing them to a single cause in an estuary where conditions are variable.

(4) Lowe A. T., Kobelt J., Horwith M., and Ruesink J., 2019: Ability of eelgrass to alter oyster growth and physiology is spatially limited and offset by increasing predation risk. *Estuaries Coasts*, **42**, 743-754.

The authors planted juvenile seed oysters and measured water chemistry inside and outside of eelgrass (*Zostera marina*) at several locations in Willapa Bay and in the Salish Sea in Washington state. They demostrate that while eelgrass modified carbonate chemistry, it did not appear to be the primary variable influencing juvenile oyster growth and survival and, instead, predation was higher and thus survival lower inside eelgrass beds where surviving oysters also grew slower.

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These authors measured growth of juvenile Pacific oyster seed deployed at numerous locations throughout Willapa Bay, and used stable isotopes to distinguish marine versus terrestrial energy sources. Results demonstrated a very distinct along estuary gradient with oysters growing faster near the mouth and slower at greater distance from the mouth and away from the mouth. Oysters also displayed distinct stable isotope ratios that reflected a strong marine signal near the mouth (-18 $\delta^{13}C$) and a stronger terrestrial signature at distance -22 $\delta^{13}C$). Oysters grown just off the bottom grew slower than those off-bottom at any given tidal elevation.

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Comparative study of the impact of environmental changes on oyster culture between USA and Japan, as collaborative research under UJNR

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Abstract: Several oyster species are cultured globally, and the Pacific oyster, *Crassostrea gigas*, is a widely cultured species in both the USA and Japan. In Japan, aquaculture production of the Pacific oyster is decreasing slightly for multiple reasons including large die-offs of adults during and after the reproductive season due to a delay in the reproductive season and poor post-spawning recovery, poor wild spat collection, and a labor shortage for both operation of aquaculture and post-harvest processing. These problems may be aggravated by environmental changes such as global warming and oligotrophication around Japan's coastal areas. The Japan Fisheries Research and Education Agency is investigating the causes of the die-off during the reproductive season and attempting to establish countermeasures. The Pacific oyster is native to Japan and was introduced to the USA for aquaculture in the early 1900's. Nonetheless, there are large differences in the culture system, habitats and environmental conditions between the two countries. A comparative study was initiated to evaluate oyster reproduction in the two countries in order to understand the effects of habitat and the environment on future success of aquaculture given predicted environmental changes. Oyster culture experiments were conducted in intertidal and subtidal zones inside and outside of seagrass habitat in Hiroshima Bay in Japan and Willapa Bay in USA during the reproductive season (March to June 2019 and February to July 2019, respectively). We focused on elucidating the effects of habitat and the environment on the energy allocation of the oyster between reproduction and somatic growth.

Key words: Pacific oyster *Crassostrea gigas*, aquaculture, die-off, energy allocation, reproduction

Background

Oyster species are cultured globally, and the Pacific oyster, *Crassostrea gigas*, is the most widely cultured species in the USA, Japan, and world. Aquaculture production of the Pacific oyster (Pacific cupped oyster in FAO, Cultured Aquatic Species Information Programme) globally exceeds 572 × 10^3 ton yr^{-1}; Japan's production was 174 × 10^3 ton yr^{-1} in 2017, and the production in USA was 26 × 10^3 ton yr^{-1} (FAO, Fishery Statistical Collections). Pacific oysters have the second highest production volume after *Crassostrea virginica* (American cupped oyster, 112 × 10^3 ton yr^{-1}) in the USA (FAO, Fishery Statistical Collections). The Pacific oyster has its origins in East Asia and has been cultivated for centuries. It has been cultured on the west coast of the USA since the 1920s, and in France since 1966. It has been widely introduced as an alternative species to indigenous oysters that had been severely depleted by overfishing and disease, and to create new industries.

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There are two large traditional production areas in Japan: Hiroshima and Miyagi Prefectures, and there are many small production areas all over Japan. Production areas are distributed from the temperate zone (western part of Japan, Kyushu) to the cold-temperate-zone (northern Island of Hokkaido). In Japan, wild spats are collected with scallop shells hung in open water areas and are widely used for aquaculture. Suspended culture from rafts or floating longlines in offshore area are the main culture system. Most of the harvested oysters are shucked for commercial distribution.

In the USA, hatchery produced spats are used as widely as wild spats. Bottom and off-bottom culture in the intertidal area (tidal flats where seagrass beds may be present) are the main culture systems. Pacific oyster culture is mainly operated in the northwestern part of the USA, primarily in the State of Washington.

In Japan, production of the oyster is decreasing gradually for multiple reasons. In addition to insufficient wild spat collection and a labor shortage for both operation of aquaculture and post-harvest processing, large die-offs of adults during and after the summer reproductive season is a major problem. This problem may be aggravated by environmental changes such as global warming and oligotrophication of coastal waters. The Japan Fisheries Research and Education Agency is investigating the causes of the die-off during the reproductive season and attempting to establish countermeasures (Hasegawa and Sakami, 2019).

Summer die-off of the oyster has also been a recognized problem on the west coast of the USA since the mid 1950's, and some of these incidences are related to stress during the reproductive season combined with environmental factors (Cheney *et al.*, 2000). However, occurrence of high mortality is less frequent in the USA than Japan. Koganezawa and Goto (1972) reported that oysters from relatively oligotrophic waters were characterized by a smaller growth rate and reduced amount of spawning eggs than oysters from eutrophic waters in Japan. Production of a large amount of eggs associated with some environmental conditions that prolong spawning may increase mortality risk. Akashige *et al.* (2006) concluded that there was a high risk of large die-offs in years with high water temperature and small amounts of rainfall, especially during the spawning season when metabolic activities are devoted to gamete production (Akashige *et al.*, 2005). Thus, excessive energy allocation to gonad development is speculated to be one of the major causes of the large die-offs.

Comparative Culturing Experiments in the USA and Japan

Elucidating the effects of habitat, environment and culture system on the energy allocation of the Pacific oyster between reproduction and somatic growth would contribute to an understanding of the reasons oysters experience challenges during the spawning season under climate change and would support establishment of countermeasures. For this purpose, comparative oyster culture experiments were conducted in high oyster production areas in the USA and Japan, where there are large differences in the culture system, habitats and environmental conditions. This study was developed and conducted as collaborative research through the UJNR bilateral. The Japan site examined was Hatsukaichi in Hiroshima Bay, a part of Seto-Inland-Sea (HI), and the USA sites were Nahcotta (NA) and Bay Center (BC) in Willapa Bay, Washington State (**Fig. 1**). In these sites, mesh bags including ten tagged oysters (oyster bag) were set up in tidal flats at intertidal and sub-tidal levels (**Fig. 2**). Oyster bags were also set up inside seagrass beds at the experimental tidal flats to evaluate the effect of seagrass, which are common around oyster culture tidal flats in the USA. In addition to the experiments around tidal flats, oyster bags were also suspended in docks and kept submerged throughout the experiments to mimic suspended culture in the subtidal zone, which is a common oyster culture system in Japan.

Gametogenic development of the Pacific oyster progresses at ambient temperature above 10℃ and reaches the peak in sexual maturation at 600℃・days heat summation (*i.e.* cumulative daily temperature exceeding 10℃; Mann, 1979 and Oizumi *et al.*, 1971). Oyster culture experiments were conducted from March to June 2019 in Japan and from February to July 2019 in the USA, which corresponds to the

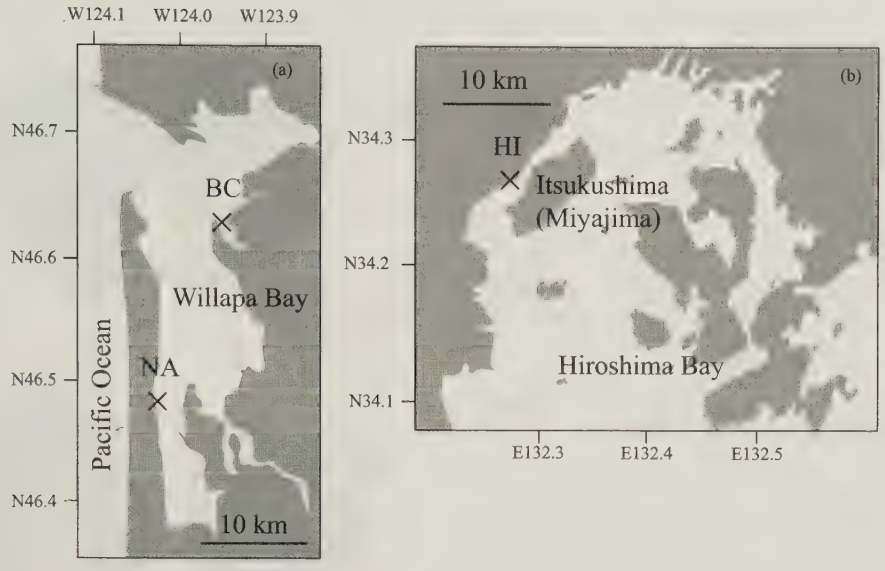

Fig. 1. Study sites for oyster culture experiments in Willapa Bay, USA (a) and Hiroshima Bay, Japan (b).

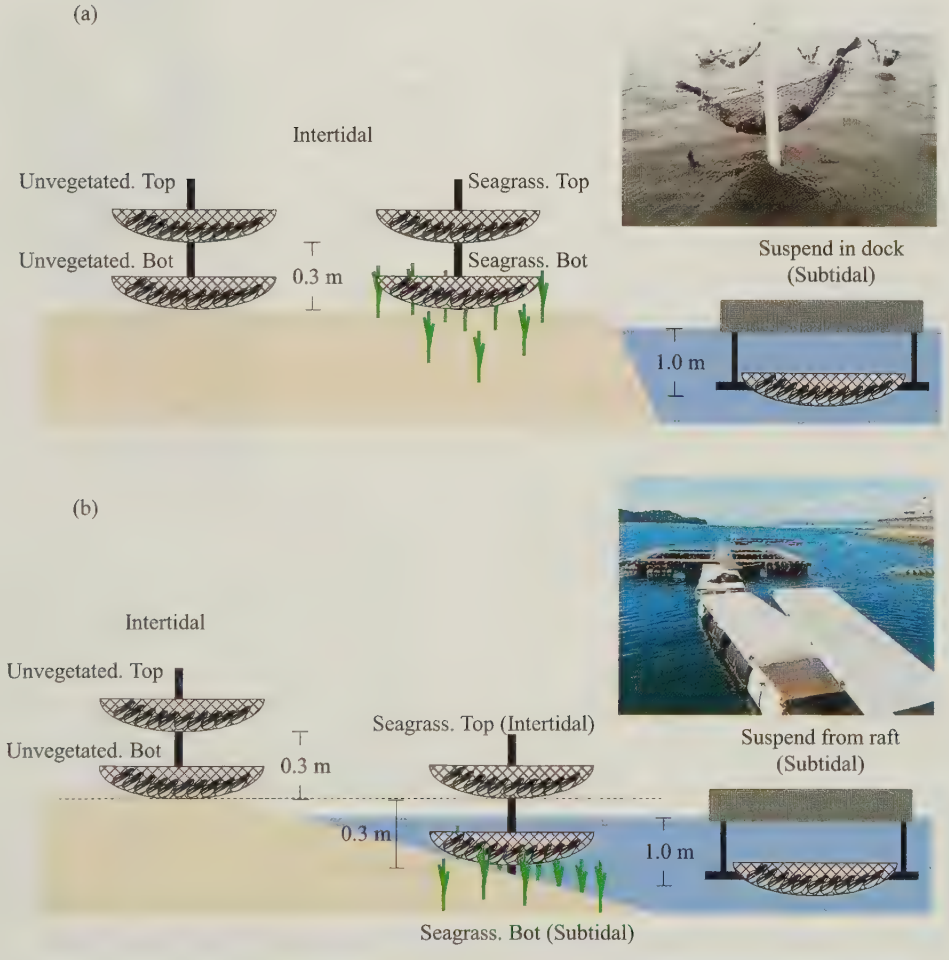

Fig. 2. Design of oyster culture experiments in Willapa Bay, USA (a) and Hiroshima Bay, Japan (b). Oyster bags, which were mesh bags including ten tagged oysters in each, were set around the tidal flat.

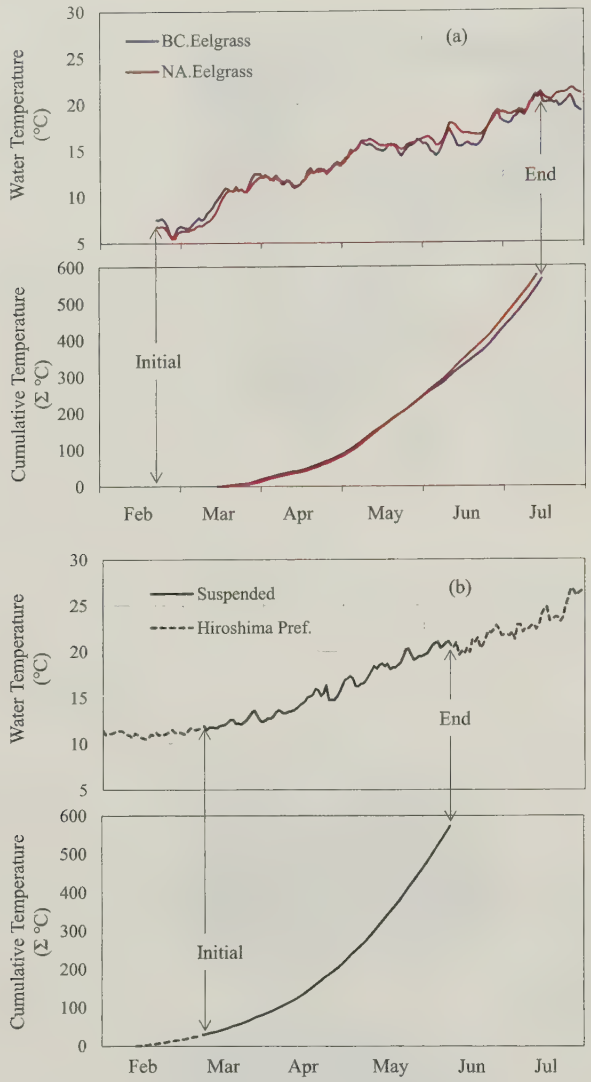

Fig. 3. Change of daily mean water temperature (during submerged times) and cumulative temperature at oyster culture experiments in Willapa Bay, USA (a) and Hiroshima Bay, Japan (b). Water temperature before and after the culture experiments obtained from Hiroshima Prefectural Fisheries and Marine Technology Center.

periods between beginning and peak (just before spawning) of sexual maturation. Peak in sexual maturation was one month later in the USA than Japan, but duration until sexual maturity (600℃ · days) was similar between USA and Japan: about 4 months (**Fig. 3**).

Oysters for the USA experiments were harvested from tidal flats at the NA site in Willapa Bay, and market-size suspended oysters were purchased from oyster farmers in Hiroshima Bay for the Japan

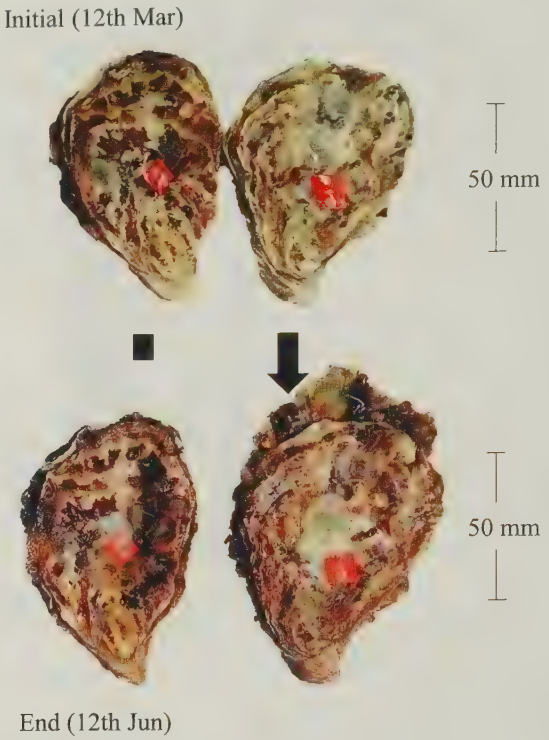

Fig. 4. Example of tagged oysters at the beginning and end of oyster culturing experiments in Willapa Bay, USA.

experiments. At the beginning of the experiments, the total wet weight inclusive of shell and shell height were measured, and the oysters were tagged for individual identification (**Fig. 4**). Initial total wet weight of oysters (shell + tissue + seawater) ranged from 77.6 to 274.6 g and from 61.9 to 162.4 g for the USA and Japan experiments, respectively. At the end of the experiments, oysters were measured for total wet weight and shell height and were also dissected. After measuring the whole tissue wet weight, the tissue was separated into the outer somatic parts (mantle, gill, and adductor muscle), inner somatic parts, and reproductive parts.

Vertical histological sections of the inner somatic and reproductive parts were prepared (**Fig. 5**). Outer somatic parts and one side of the inner parts were weighed and stored frozen at −20℃. Frozen samples were then freeze-dried and homogenized for nutrient component analysis (crude carbohydrate, protein and fat). The other side of the inner parts were weighed and fixed in Davidson's fixative, dehydrated in tissue dehydration solutions and embedded in paraffin. The paraffin blocks were

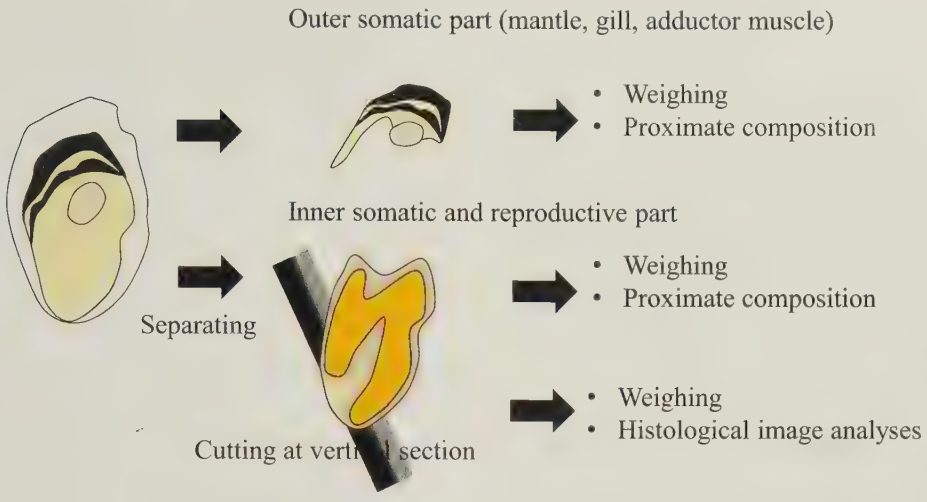

Fig. 5. Workflow of oyster sampling for evaluating energy allocation to the gonads.

Fig. 6. Example of tissue section of oyster inner parts (male) stained with hematoxylin and eosin.

sliced at 5–8 μm thickness and stained with hematoxylin and eosin (**Fig. 6**). Sectional area ratio of gonad to total inner part were histologically determined and volume ratio, which are substitutes for weight ratio, were calculated. Total carbohydrate content of dried sample was determined using the phenol-sulfuric acid method (Dubois *et al.*, 1956). The amount of total nitrogen in samples was measured using an elemental analyzer (Flash EA1112, Thermo-Finnigan) and nitrogen content was converted to protein content by multiplying the conventional conversion factor of 6.25. Crude fat content was determined by the modified gravimetric method (Ichihara *et al.*, 2011) based on the method originally described by Bligh and Dyer (1959). For total lipid extraction, tert-butyl methyl ether was used instead of chloroform. Energy contents of each tissue part were calculated as the sum of the energy content in each nutrient component estimated by the following factors: carbohydrate (17.2 kJ/g DW), protein (23.9 kJ/g DW) and lipid (39.8 kJ/g DW) (Ansell, 1974). Finally, energy allocation to the gonads was collectively estimated with the obtained data.

Relative growth rate (RGR) of shell height during culture experiments was calculated as follows:

$$\mathrm{RGR} = \frac{Ln\,(Shell\ height_{end}) - Ln\,(Shell\ height_{initial})}{Culture\ period\ (day)}$$

Condition index (CI) was calculated as follows;

$$\mathrm{CI} = \frac{TSW}{TTW - SLW} \times 10^2$$

where TSW is the tissue weight (gWW), TTW is the total weight with shell (filled with water, gWW) and SLW is the shell weight (gWW).

This is a progress report of the UJNR collaborative study. At the moment, the culture experiments and sample analysis have been finished, and collected data are being organized and analyzed. The results

are planned to be published in future reports.

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Mass mortality in the Pacific oyster, *Crassostrea gigas* occurred in 1979, 1994, 2001 and 2002, and relationships with surface water temperature or rainfall in Hiroshima Bay were analyzed using a dataset from 1970 to 2004. Mass mortality occurred in years with high water temperature (*i.e.* the number of days over 20℃ was greater than the mean+SD) and small rainfall (*i.e.* cumulative total rainfall from July to October was smaller than the

mean－SD) except in 2001. Spat introduced from Miyagi Prefecture experienced extraordinary mass mortality in 2001, which has a colder climate than Hiroshima Prefecture. Therefore, there is high risk of mass mortality of local oysters in years with high water temperature and small rainfall.

(2) Akashige, S., Hirata Y., Takayama K., and Soramoto K., 2005: Seasonal change in oxygen consumption rates and filtration rates of the cultured Pacific oyster *Crassostrea gigas*. *Nippon Suisan Gakkaishi*, **71(5)**, 762-767.

The oxygen consumption rate (OCR) and filtration rate (FR) of cultured Pacific oysters, *Crassostrea gigas*, of different sizes were measured in still water systems at different seawater temperatures in various seasons. The OCR had clear relationships with water temperature (t ℃) and dry body flesh weight (W_d g) expressed by the formula: OCR (mg O^2 hr^{-1} ind^{-1}) = $(0.072t-0.64)$ $W_d^{0.75}$ and there was no difference among seasons. The FR was expressed by the formula: FR (L/h/ind) = $(0.70t-6.6)$ W_d in non-spawning seasons. However, in the spawning season, the FR was expressed by the formula: FR = 4.9 W_d, which was lower than the value expected by the formula applied during the non-spawning season. These results clearly showed that feeding activities declined during the spawning season, but oxygen demand (weight base and temperature dependent) was constant throughout the year. Oysters would be especially vulnerable during the spawning season when metabolic activities would be devoted to gamete production.

(3) Cheney, D.P., MacDonald B.F., and Elston R.A., 2000: Summer mortality of Pacific oysters, *Crassostrea gigas* (Thunberg): Initial findings on multiple environmental stressors in Puget Sound, Washington, 1998. *Journal of Shellfish Research*, **19**, 353-359.

Summer mortality has been a recognized problem for oyster aquaculture on the west coast of the USA since the mid 1950's, and these authors related some of these incidences to stress during the reproductive season combined with environmental factors. Nonetheless triploid oysters experienced higher mortality than diploids.

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Oyster aquaculture using seagrass beds as a climate change countermeasure

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Abstract: In the framework of the Sustainable Development Goals (SDGs) led by the United Nations, coastal management methods are required to achieve both sustainable food production and environmental conservation as a climate change countermeasure. Oyster farming is an important food production method now being developed in coastal areas around the world. Recently, climate change has caused several negative effects on oyster aquaculture such as poor spat collection due to oligotrophication, ocean acidification, and poor spat growth and survival due to frequent anoxic events derived from high seawater temperature. The oysters cultivated in many regions of the world are intertidal species inhabiting intertidal zones such as sandy/muddy tidal flats and estuaries, where seagrass beds are often distributed in adjacent lower intertidal and subtidal areas. Seagrass vegetation is one of the most important ecosystems functioning as a countermeasure for global climate change. Not only does it mitigate greenhouse gas emissions by sequestration and storage of blue carbon derived from atmospheric CO_2, but it also functions as an adaptation measure providing a buffering function against ocean acidification and water quality improvement.

Based on the concept of aquaculture supported by natural ecosystem interactions between oysters and seagrass beds, our project examined whether aquaculture techniques that take into account both mitigation and adaptation to climate change are effective for both sustainable use of coastal areas and environmental conservation. We conducted field experiments in both the French Mediterranean Sea and the Seto Inland Sea of Japan to clarify the effect of eelgrass beds on (1) natural oyster spat collection and (2) growth and survival of oyster spat. The results of our experiments revealed that spat recruitment was significantly higher in areas without eelgrass distribution, while spat growth and survival rate after the settlement were significantly higher in eelgrass beds even when anoxic events occurred in the study areas. Therefore, our results indicate a possibility that seagrass vegetation contributes to sustainability of oyster aquaculture by mitigating environmental degradation during cultivation.

Keywords: oyster aquaculture, *Zostera*, *Crassostrea gigas*, blue carbon ecosystem, integrated coastal management

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Introduction

As the impacts of climate change on human societies and ecosystems intensify, various measures to address climate change are being implemented in various fields across the world. The Paris Agreement adopted in 2015 by the UN Conference of the Parties to the United Nations Framework Convention on Climate Change (UNFCCC) states that efforts will be made to keep global average temperature rise well below 1.5°C compared to before the Industrial Revolution. To achieve the climate target, it is recommended that the balance between greenhouse gas emissions and absorption be reduced to zero. However, the CO_2 emissions that are currently in operation and the CO_2 emissions that are committed by fossil-fuel energy infrastructure were estimated at more than 800 Gt in 2018, which is already far beyond the targets of the Paris Agreement to keep the temperature rise below 1.5°C (Tong *et al.*, 2019). Therefore, in order to meet the Paris Agreement climate goals, it will be necessary to retire these infrastructures as soon as possible, as well as increase CO_2 sequestration sinks.

Moreover, climate change is triggering food crises in various regions of the world, and the measures for sustainable food production are also urgently needed. It is generally recognized that there is a trade-off between current food production systems and climate change mitigation measures (Elmqvist *et al.*, 2013). For example, deforestation in terrestrial ecosystems is accelerating to create pastures and other agricultural fields to increase food production (Schiermeier 2019). Therefore, some countermeasures are needed to harmonize sustainable food production with climate change mitigation (Bommarco *et al.*, 2013).

Shallow coastal ecosystems are now highlighted as one of the measures to mitigate the effects of climate change (Hoegh-Guldberg *et al.*, 2019). In particular, blue carbon ecosystems such as mangroves, salt marshes and seagrass vegetation are important ecosystems currently being considered as a new and effective atmospheric CO_2 sink (Kuwae and Hori, 2018). In addition, the blue carbon ecosystem is known as one of the most productive ecosystems with high biodiversity resulting in higher food production. Therefore, the blue carbon ecosystem is a typical ecosystem providing co-benefits to both sustainable food production and climate change mitigation.

Oyster farming is an important food production method now being developed in coastal areas around the world. Recently, climate change has caused several negative effects on oyster aquaculture such as poor spat collection due to oligotrophication, ocean acidification, and poor spat growth and survival due to frequent high seawater temperature and anoxic events (Hori *et al.*, 2018; Lagarde *et al.*, 2018, 2020). The oysters are originally intertidal species inhabiting intertidal zones such as sandy/muddy tidal flats and estuaries, where seagrass beds are often distributed in adjacent lower intertidal and subtidal areas. Not only can seagrass vegetation mitigate greenhouse gas emissions through sequestration and storage of blue carbon derived from atmospheric CO_2, but it also functions as an adaptation measure buffering against ocean acidification and improving water quality (Larkum *et al.*, 2006; Duarte *et al.*, 2013; Groner *et al.*, 2018).

Based on the concept of returning to traditional aquaculture using the natural ecosystem interactions between oysters and seagrass beds (Hori *et al.*, 2018), our project is now demonstrating whether aquaculture techniques that take into account both mitigation and adaptation to climate change are effective for both sustainable use of coastal areas and environmental conservation. In this study, we present the results from field experiments to clarify the effect of eelgrass beds on the sustainability of oyster aquaculture in both the French Mediterranean Sea and the Seto Inland Sea of Japan. The seagrass contribution to oyster production was divided into two processes in relation to oyster life cycle stages: recruitment processes from the larval stage to spat settlement, and post-recruitment processes with spat growth. A series of *in situ* experiments were conducted in Japan and France (1) to demonstrate the larval recruitment variability in shellfish farming areas in the presence or absence of eelgrass beds, and (2) to reveal the effects of eelgrass vegetation on the survival and growth of oyster spats. To our knowledge, there has been no prior case study directly demonstrating the effect of oyster-seagrass

interactions on ecosystem functioning, although there has been some prior modelling research on material cycling in a coastal ecosystem including oyster and seagrass beds (*e.g.* Kishi and Oshima, 2008).

Methods

Study sites

The Seto Inland Sea (coordinates at its centre: 34.1667 N, 133.3333 E) in Japan and the Thau lagoon (coordinates at its centre: 43.41 N, 3.6241 E) in France were chosen as study areas for this research (**Fig. 1**). The Seto Inland Sea is located at the southwestern part of the main island of the Japanese archipelago. Rafted aquaculture using natural spats of the native Pacific oyster *Crassostrea gigas* is flourishing in many areas of the Seto Inland Sea. The production in the Hiroshima Bay and all areas of the Seto Inland Sea accounts for about 60% and 80% of the national production of oysters in Japan, respectively. Recent oligotrophication due to legal restrictions on nutrient input from the watershed has resulted in eelgrass recovery in the Seto Inland Sea over the last decade. It has been estimated that the area of seagrass meadows has increased from 6000 ha to about 10,000 ha in 2011 (Hori and Tarutani, 2015).

The Thau lagoon is the largest lagoon located on the southern French coast in the Mediterranean Sea. The lagoon is famous for oyster farming using non-native Pacific oyster spats attached on ropes containing a specific cement. The ropes with the spats are hung from oyster tables established in

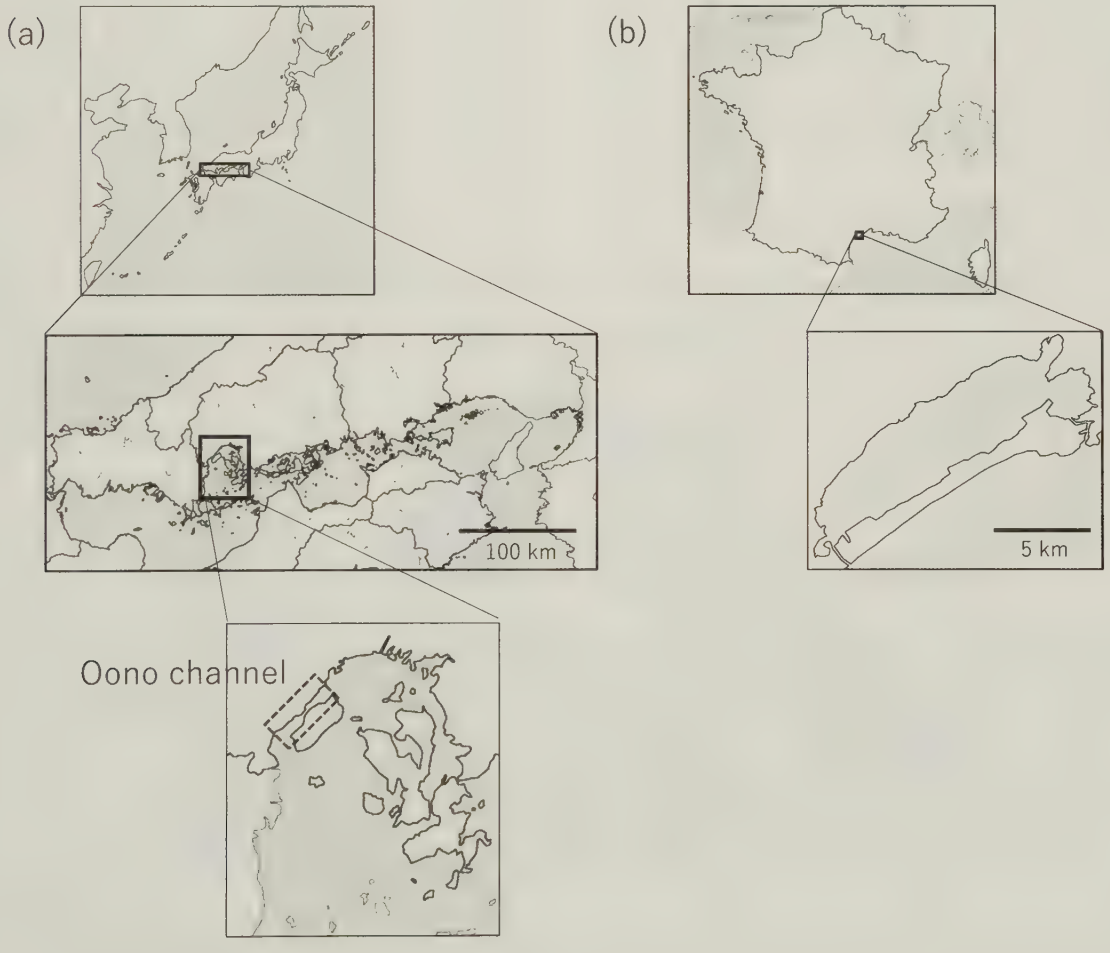

Fig. 1. The two comparative study sites, including a) Seto Inland Sea, including the Hiroshima Bay and Oono channel, and b) Thau Lagoon near the Gulf of Lion, Mediterranean Sea. These maps were revised from Hori *et al.* (2018).

the nearshore zone. About 10% of the French national production of oysters is cultivated there, representing the largest oyster farming area in the Mediterranean Sea. It has been suggested that the recovery of eelgrass beds is still proceeding, and that now the area of seagrass distribution extends up from 2 ha to 800 ha (Hori, personal communication with Syndicat mixte du bassin de Thau). The expansion of eelgrass meadows was observed even within oyster farming areas in June 2016.

1. Larval recruitment variability in shellfish farming area

In Thau lagoon, settlement and recruitment of oysters were monitored at six contrasting stations in August 2017 from east to west, in the absence or presence of *Zostera* meadow and shellfish farming in the Thau Lagoon (**Fig. 1**). The oyster collectors were deployed as described by Lagarde *et al.* (2017; 2019) (**Fig. 2**). The collectors were deployed in the water column (**Fig. 3a**) inside/outside shellfish farm sites and also hung from the oyster tables above and below the canopy in *Zostera* meadows inside *Zostera* spp. sites (**Fig. 3b**) (Lagarde *et al.*, 2020). All collectors were sampled after 2 weeks of immersion to assess pediveligers and postlarvae abundance and after 4 weeks of immersion to assess oyster spat abundance.

In Japan, oyster spat collectors were deployed on the seagrass beds and also hung from the oyster rafts in the Oono channel of the Hiroshima Bay in July 2017 (**Fig. 1**). Only the collectors in seagrass beds were exposed daily to the atmosphere due to tidal movement. All collectors were sampled after 4 weeks of immersion to assess oyster spat abundance.

2. Effect of eelgrass vegetation on oyster survival and growth

Our hypothesis was that seagrass beds could maintain or improve the safety and environmental sanitary conditions for oysters, especially reduction of harmful microbiomes (*e.g.* Lamb *et al.*, 2017; Groner *et al.*, 2018). To clarify the effects of seagrass vegetation on microbiome in both oyster and ambient sea waters, a series of census and experiments were carried out in the Thau Lagoon

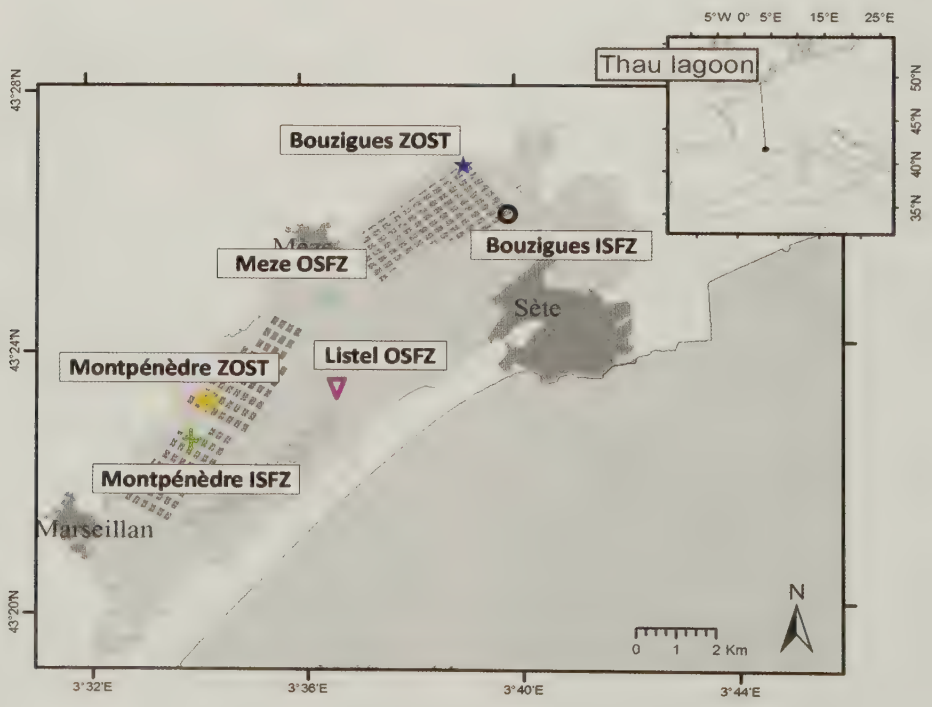

Fig. 2. The six sampling sites scattered from east to west inside Thau Lagoon with three conditions (ISFZ; Inside Shellfish Farming Zone, OSFZ: Outside Shellfish Farming zone, Zost: *Zostera* spp. beds). Shaded squares located at northwest side of the lagoon show oyster tables. This map was revised from Lagarde *et al.* (2020).

in 2017 and 2018 in the presence and absence of *Zostera* meadows (**Fig. 2**: Bouzigues ISFZ, Bouzigues ZOST, Montpénèdre ZOST, Montpénèdre ISFZ) for analyzing environmental DNA and oyster microbiome. The analyses are still ongoing, therefore the results are not shown in this paper. In parallel, the effect of *Zostera* meadows on growth and survival of juvenile oysters after 3 months of growing (September-December) was tested inside and outside eelgrass beds at Bouzigues and Montpénèdre in the Thau Lagoon in 2018 (**Fig. 2**).

In Japan, we established a field experiment in Hiroshima Bay to clarify the contribution of eelgrass beds to the survival and growth of oyster spats as a feasibility study of oyster-seagrass interactions (Hori *et al.*, 2018). We established an experimental area (5 m×5 m) in the lower intertidal area on the tidal flat with seagrass vegetation, and set a raft floating on the sea surface 200 m offshore from the tidal flat, hanging a replicate of three cages at a depth of 2 m from the sea surface using vinylon ropes. Thirty spats of each of three native species (*C. gigas*, *C. nippona* and *C. sikamea*), which were hatched from the same lot, were put into the cages on the tidal flat, and the other half of the spats of each species were put into the cages hanging from the raft. The experiment was conducted for two months from November 2016 to January 2017. In addition, we took environmental DNA and microbiome samples from the oysters and ambient waters in both tidal flat with seagrass and the raft to clarify the effects of seagrass vegetation on the sanitary condition of the oysters. Analyses of this data and the results from Thau lagoon are ongoing.

Result and Discussion

1. Larval recruitment variability in shellfish farming area

In France, the abundance of young settlers was lower within the canopy of eelgrass beds than above the eelgrass canopy at both *Zostera* sites (**Fig. 4**). The best sites for settlement were the OSFZ sites (Listel and Meze: outside of the oyster farming and seagrass areas), which confirm those of Lagarde *et al.* (2017; 2018; 2019), where there is a combination of high level hydrodynamic connectivity and favorable trophic supply. These results indicate that eelgrass beds are unlikely to be a preferred site for oyster settlement over other nearby sites in term of abundance. However, between the sites with seagrass vegetation, the abundance of young settlers was much higher in the Bouzigues site where the abundance of eelgrass vegetation was also higher than that in the Montpénèdre site. (Hori, unpublished data). Although eelgrass vegetation does not increase natural spat recruitment, there may be no negative effect on the recruitment.

In Japan, the mean abundance of recruited spats was lower in the eelgrass beds than that hung from the raft without eelgrass vegetation (**Fig. 5**), although the difference was not statistically

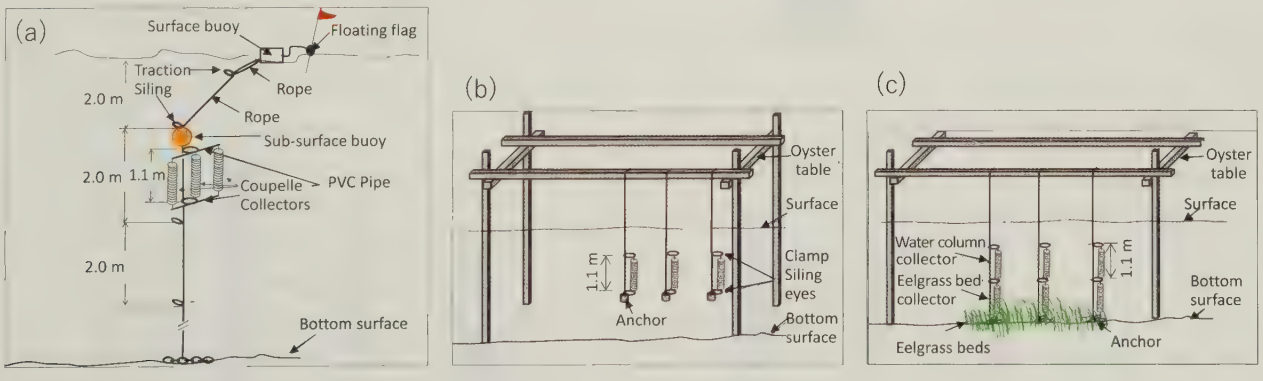

Fig. 3. Oyster spats collectors deployed in each site of Thau lagoon. In the site of Outside Shellfish Farming Zone (OSFZ), mooring system (a: left) was used. In the site of Inside Shellfish Farming Zone (ISFZ) without eelgrass beds (b: middle) and the site of inside shellfish farming zone with eelgrass beds (ZOST, c: right), collectors were hung from the oyster table, and deployed above eelgrass canopy (water column) and below the eelgrass canopy. These illustrations were revised from Lagarde *et al.* (2020).

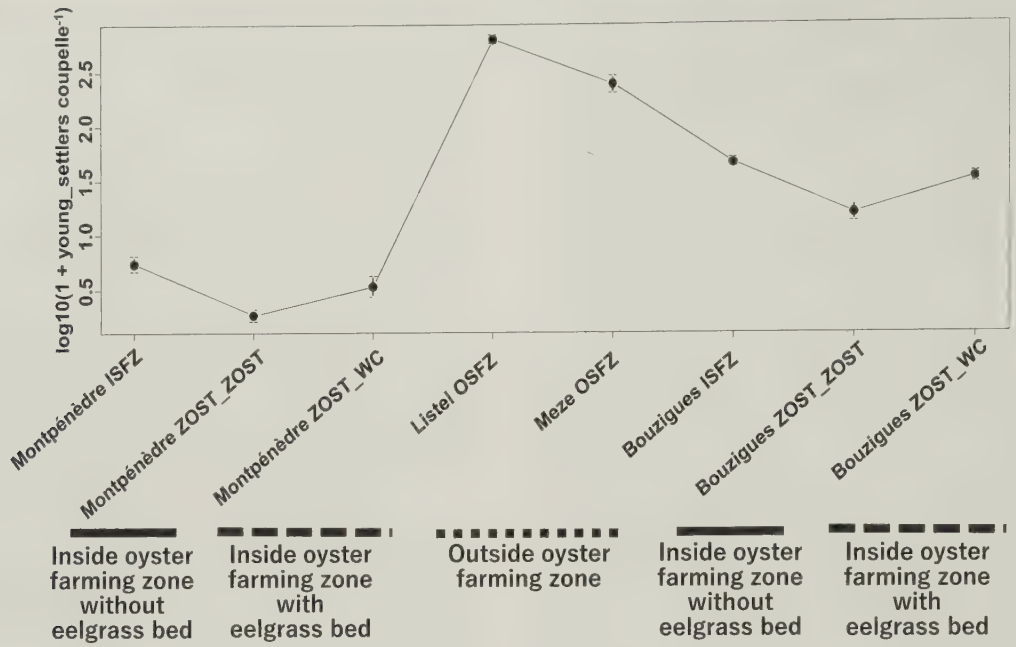

Fig. 4. The abundance of young settlers (pediveligers and postlarvae) on each coupelle collector (mean ± SE) in each site. ISFZ: Inside Shellfish Farming Zone, OSFZ: Outside Shellfish Farming Zone, ZOST_ZOST: collectors inside eelgrass bed, ZOST_WC: collectors in water column above eelgrass bed.

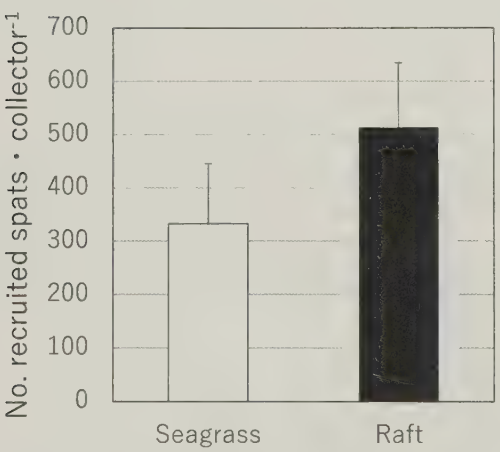

Fig. 5. The abundance of recruited spats on each collector (mean ± SD) on both eelgrass beds and oyster raft.

significant (ANOVA: F=3.573, p>0.05). Natural oyster spats are generally collected in the intertidal zone where native spats of *C. gigas* most frequently settle (FRA, 2016), and young settlers reach the intertidal zone during high tide. The depth of the collectors hung from the raft is similar to the depth of the intertidal zone during high tide.

2. Effect of eelgrass vegetation on oyster survival and growth

Although eelgrass vegetation has no significant positive effect on oyster recruitment, the results of spats cultivation experiments exhibited that eelgrass beds seemed to develop a better environment for spat survival and growth in both France and Japan. In France, the survival rate of oyster spats after three months of cultivation was higher in the sites with eelgrass beds (**Fig. 6a**). Moreover, the survival rate was higher in the Bouzigues site with dense eelgrass beds than the Montpénèdre site with sparse eelgrass beds, suggesting that more abundant eelgrass vegetation could be a better environment for oyster spats. The spats also exhibited better growth in shell length and fresh weight in the site with eelgrass beds than the site without eelgrass beds after three-months cultivation (**Fig. 7a**).

In the experiment conducted in Hiroshima Bay, the survival rate of oyster spats was higher in the tidal ground with eelgrass beds than that hung from the raft, except for *C. nippona* (*iwagaki*) spats (**Fig. 6b**). The tidal flat may not be a good habitat for the *iwagaki* oyster because this species naturally

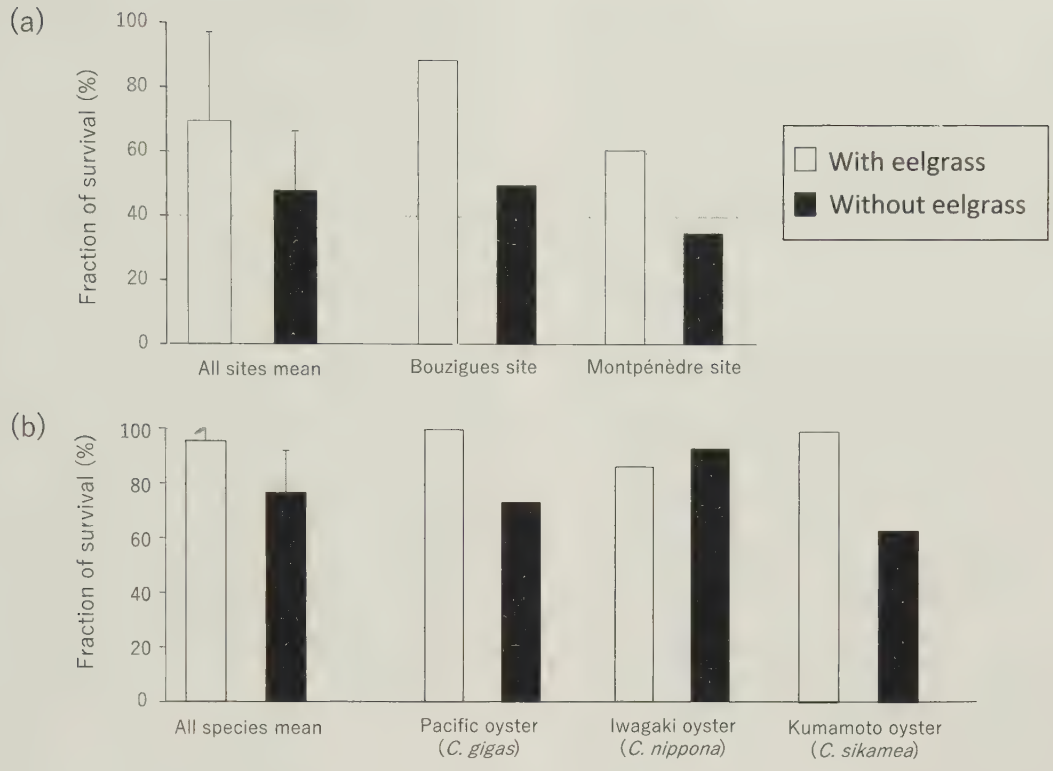

Fig. 6. The survival rate of oyster spats in a) Thau lagoon and b) Hiroshima Bay. In Thau lagoon, the biomass (shoot density x shoot height) of eelgrass in Bouzigues site was much larger than that in Montpénèdre site.

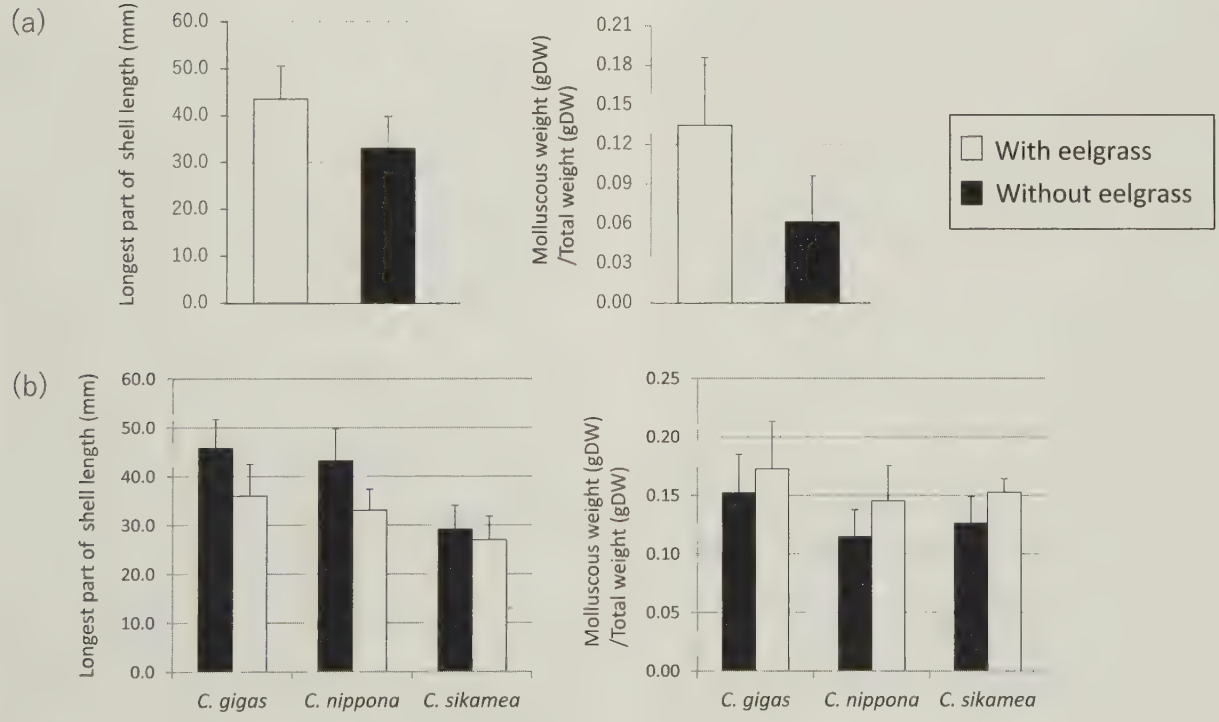

Fig. 7. The difference in shell length and relative meat weight between the site with eelgrass beds and the site without eelgrass beds as an index of growth rate in a) Thau lagoon and b) Hiroshima Bay. The results in Hiroshima Bay was originally referred from Hori *et al.* (2018).

inhabits subtidal rocky shore without exposure to the atmosphere. The growth rate based on relative fresh weight per total weight after two months was also better in the tidal flat with seagrass beds (**Fig. 7b**), although the shell length of all three species were shorter in the tidal flat with eelgrass. Oyster spats in the cages deployed on the tidal flat were frequently exposed to heavy wave action, such as fishing boat wake during the low tide period, and the tip of the shells were often broken by clashing each other in the cage (Hori, personal observation). This may be a reason that the shell length was shortened in all spats in the tidal flat site.

At least two possible hypotheses can be raised for seagrass vegetation providing better environment to oyster spats: trophic support and the improvement of the sanitary conditions. As a trophic support hypothesis, Hori *et al.* (2018) suggested that oyster spats cultivated in the tidal flat could be used for pelagic production and benthic production in eelgrass beds based on the result of carbon and nitrogen stable isotope analyses. Even *iwagaki* oyster spats with poor survival exhibited better growth in relative fresh weight, thus trophic function of eelgrass beds may have some contribution to the spat growth. These results are also supported in study of Barbier *et al.* (2017) showing that bivalve recruitment abundance is lower in *Zostera marina* beds than others benthic habitat, like subtidal coarse sand or maerl beds (Corallinophycidae, Rhodophyta). However, they observed higher accumulation of fatty acids in the digestive glands of juveniles settled in *Z. marina,* suggesting higher food availability. However, *Crepidula fornicata* banks appear to be the favorable habitat for bivalve recruitment (higher recruit abundance and diversity) with individuals having fatter digestive glands.

The other hypothesis is that seagrass beds could maintain or improve the safety and environmental sanitary conditions for oysters (*e.g.* Lamb *et al.*, 2017; Groner *et al.*, 2018). There are some known negative environmental factors. Some lethal viruses and bacterial pathogens capable of causing mortality of oysters are well known, and anoxia events are a growing threat related to seawater temperature rise during the summer. Lamb *et al.* (2017) reported that the presence of seagrass vegetation reduced 50% of the relative abundance of potential bacterial pathogens capable of causing disease in humans and marine organisms. In our ongoing analyses of the environmental DNA and microbiome from the oysters and ambient waters, some of the results support the previous report by Lamb *et al.* (2017), although more detailed analyses are needed.

Anoxia decreases the growth of oysters even when it is not lethal, because oysters keep their shells closed in an anoxic environment. In fact, serious anoxia occurred around oyster farming areas in Thau lagoon in August 2018 (Author's personal observation). The exceptional climatic conditions in 2018 led to a heat wave and summer anoxia in Thau lagoon, causing massive mortality of oysters and mussels (4000 t, corresponding to 41% of the annual production). Even in this condition, the survival rate of oyster spats in the site with more abundant eelgrass vegetation was approximately 90%, suggesting a possibility that eelgrass vegetation mitigated anoxic environment in the study site. To unravel this mitigative function, more surveys and analyses are needed, such as the relationship between mortality and dissolved oxygen concentration in detail.

In conclusion, seagrass vegetation has potentially positive effects on oysters as reported in Morimoto *et al.* (2017). Our results directly revealed that eelgrass beds can contribute to some processes of oyster aquaculture such as spat cultivation. Therefore, oyster aquaculture using oyster-seagrass interactions could be an effective coastal management option to achieve both sustainable food production and environmental conservation as a climate change countermeasure. For example, oyster-eelgrass interactions may support high water transparency and better sanitary conditions, which are also beneficial for recreational uses. Larger eelgrass beds can absorb more carbon dioxide from the atmosphere and store them as organic carbon (Kuwae and Hori, 2018), which can mitigate ocean acidification and, moreover, offset the carbon emissions from oyster aquaculture and recreational activities. Such local offset systems for carbon emissions can contribute towards solutions for climate change in relation to the framework of the Sustainable Development Goals (SDGs) led by the

United Nations.

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Annotated Bibliography of Key Works

(1) Hori M., Hamaoka H., Hirota M., Lagarde F., Vaz S., Hamaguchi M., Hori J., Makino M., 2018: Application of the coastal ecosystem complex concept toward integrated management for sustainable coastal fisheries under oligotrophication. *Fish. Sci.*, **84(2)**, 283–292.

Harmonizing coastal fisheries with water quality improvement has become an essential factor for the sustainable use of coastal ecosystem services. Here, we present the scope of our study based on an interdisciplinary approach including ecological, socio-economic and socio-psychological actions. We chose to focus on the interaction between oyster aquaculture and seagrass vegetation as a typical ecological action using the coastal ecosystem complex (CEC) concept. Coastal organisms have adapted their traits to the environment over a long period of time, so that restoration of the CEC represents reconstruction of the original process of coastal production. Subtidal seagrass vegetation with intertidal oyster reefs is the original CEC in Japan, which would be expected to enhance coastal production by improving the production efficiency without adding nutrients. A simple field experiment examining carbon and nitrogen contents and stable isotope ratios revealed that oyster spats cultivated on a tidal flat adjacent to seagrass beds had higher nitrogen contents and higher $\delta^{13}C$ ratios than spats cultivated in an offshore area using only pelagic production. This result suggests that utilization of the CEC, which enables oysters to use both pelagic and benthic production, has potential to sustain a food provisioning service for humans, even in oligotrophic conditions.

(2) Lagarde F., Richard M., Bec B., Roques C., Mortreux S., Bernard I., Chiantell C., Messiaen G., Nadalini J., Hori M., Hamaguchi M., Pouvreau S., d'Orbcastel E. R., and Trenblay R., 2018: Strophic environments influence size at metamorphosis and recruitment performance of Pacific oysters. *Mar. Ecol. Prog. Ser.*, **602**, 135–153.

Reproduction and recruitment of benthic invertebrates are influenced by climate and by the ecological structure of marine ecosystems, along with local anthropogenic pressures such as eutrophication or oligotrophication. Using the Pacific oyster, *Crassostrea gigas*, as a biological model, we tested the hypothesis that the variability in prodissoconch II (PII) size (*i.e.* size at metamorphosis) depends on

ecological functioning. Settlement and recruitment were assessed at 5 sampling sites in the French Mediterranean shellfish farmed Thau lagoon during the main summer recruitment events in 3 consecutive years (2012–2014). Hydrobiological and planktonic analyses were conducted at 3 sampling sites. Our results showed that recruitment was extremely heterogeneous, ranging from 0 to 260 ± 27 SE ind. dm^{-2} throughout the ecosystem and was linked with variability in PII size, which ranged from 180 to 296 μm. The annual temporal pattern of PII sizes appeared to be controlled by temperature during the settlement period, whereas the spatial pattern depended on phytoplankton biomass and on the trophic functioning of the ecosystem. Smaller PII sizes were significantly correlated with the highest phytoplankton biomass, while larger PII sizes were positively correlated with mixotrophic cryptophyte abundance. We found an inverse relationship between PII size and survival after metamorphosis, showing that recruitment success was associated with smaller PII sizes. Regional climate conditions and local trophic functioning appear to be key factors in metamorphosis and consequently contribute to recruitment heterogeneity. Further studies should be performed in other ecosystems following an oligotrophication trajectory to generalize this result.

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Kelp, *Saccharina* spp, population genetics in New England, US, for guiding a breeding program of thermally resilient strains

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Abstract: The cold-water sugar kelp, *Saccharina latissima* has a circumboreal distribution and in the Northwest Atlantic is at its southern distributional limits in Long Island Sound. An understanding of genetic diversity of natural kelp populations is critical for making recommendations for breeding and cultivation efforts of the growing seaweed aquaculture sector in the US. An important component of the ARPA-E's MARINER project is selectively breeding *Saccharina* spp. in order to improve overall productivity for biofuels, feeds and food.

Historical records indicate the presence of regional kelp ecotypes based on physiological tolerance, specifically temperature. We made collections of 15 wild *Saccharina* spp. populations via SCUBA along the New England coast. Microscopic gametophytes were isolated and the parental populations were used to make over 500 hybrid crosses that were planted at several farm locations over several years. We then used genome-wide single nucleotide polymorphism data to explore the genetic structure of the kelp throughout this region. An assessment of the sequence diversity revealed distinct genetic variation between the Gulf of Maine and Southern New England (F_{ST} > 0.25), confirming that Cape Cod acts as a barrier to *S. latissima* gene flow. Furthermore, based on the analysis of molecular variance (AMOVA), we found the largest variance (58%) within sites. We also observed admixture among three ancestral populations and isolation by distance. Future steps for this project include skim sequencing the haploid microscopic gametophytes to identify trait heritability, phenotypic diversity observed for both morphological traits and tissue composition, and genomic selection. Furthermore, in the future, we plan to place our sequence data into a larger context to include samples from sites in the east Atlantic and Pacific Oceans.

Key words: *Saccharina*, population genetics, breeding, thermal resilience

Introduction

For centuries, humans have harvested different seaweeds for food, medicinal purposes, feed, and more recently as raw materials for industrial processes (Augyte *et al.*, 2018; Grebe *et al.*, 2019; Kim, *et al.*, 2019). Over the years, with human population rise, seaweeds have become increasingly important in global food security. Worldwide, seaweed aquaculture is an important component of total marine aquaculture production, and is experiencing exponential growth in the last 50 years (Kim *et al.*, 2014; Augyte *et al.*, 2017; Langdon *et al.*, 2019; FAO, 2020). Compared to Asian countries including China, Korea, Japan, Indonesia and the Philippines, seaweed aquaculture is a young

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industry in the United States of America (Kim *et al.*, 2019). It is estimated that the U.S. Farmed seaweed production in the year 2019 was 249,000 – 272,000 kg wet weight with 80% of this production coming from *Saccharina latissima* or sugar kelp (Piconi *et al.*, 2020). It is estimated that edible seaweed production will double in the next five years and will increase by four times by 2035 (Piconi *et al.*, 2020).

Various studies have addressed the gametophyte growth, propagation, and thermal tolerance, as well as ecosystem benefits of seaweed cultivation in the open – ocean (Egan and Yarish, 1988; Kim *et al.*, 2014; Augyte *et al.*, 2017; 2018; Wade *et al.*, 2020), however, a lot remains to be done to achieve a deep understanding of kelp ecophysiology and application to cultivation. To meet the growing demand for increased seaweed aquaculture production, the MARINER program funded by the U.S. Dept. of Energy is focused on increasing production for biofuels, animal feed and human food. As interest in commercially viable species continues, it is therefore critical to have baseline information regarding the underlying genetics of the wild populations to guide sustainable practices. Furthermore, an important component of breeding superior cultivars is selecting kelp strains that are high yielding, and disease and thermally tolerant. The purpose of this study was to assess the genetic structure of wild *S. latissima* populations in Northwest Atlantic, from the southern range of its distribution, throughout Southern New England to the Gulf of Maine. The results of this study will inform managers and conservation groups on genetic hotspot areas of diversity, connectivity and gene flow. Finally, this and future research can guide breeding and cultivation efforts of wild *S. latissima* by identifying phenotypic variation and hybrid vigor.

Materials and Methods

Collections of 15 wild *Saccharina* spp. populations via SCUBA along the coast of New England, in the Northwest Atlantic were made. Commercially important morphometric measurements were done to characterize phenotypic variation specifically on blade length, width, and thickness and stipe length and thickness. Tests were run to correlate environmental variables with morphology. Samples were collected at peak reproduction and sorus material was isolated and meiospores were released to cultivate the microscopic stage of the life cycle, specifically the male and female gametophytes. These gametophytes were used to make over 500 hybrid crosses that were out planted at several aquaculture farms over two years. Parental sporophytic blade material was collected for DNA extraction and were used for genotyping at the Diversity Arrays Technology (DArT) facility in Canberra, Australia. Reduced genomic representation was generated using restriction enzymes and Ilumina HiSeq2500 and then reads were processed and single nucleotide polymorphisms (SNPs) were called using proprietary DArT analytical pipelines (Kilian *et al.*, 2012). Genome-wide SNP data coupled with AMOVA (Analysis of Molecular Variance), FST, admixture, isolation by distance and PCoA (Principal Component Analysis) were used to explore the genetic structure of kelp throughout the region.

Results

Large morphological variation was observed across all 15 locations. For example, adult kelp blade lengths ranged from 84.5 cm – 227 cm; blade widths from 3.4 mm – 41.4 mm; blade thickness ranged from 0.8 – 2.28 mm; stipe diameter from 2.17 mm – 14.43 mm; and stipe length from 4.8 cm – 122.7 cm.

The populations within the Gulf of Maine showed significantly higher genetic diversity compared to populations in the southern region, in the Southern New England area. Results further indicate that roughly half (56%) of the total variation exists within locations (AMOVA, p-value<0.001). Isolation by distance, indicated by positive correlation between genetic and geographic distances, was observed for both the Gulf of Maine (r=0.47, p-value=0.002) and Long Island Sound (r=0.94, p-value=0.125). The pair-wise genomic F_{ST} between the two regions was >0.25 and was supported by the PCoA plots. Despite the isolation by distance pattern in each region, ancestry analyses indicate a complex history of historical gene flow both within and between the regions stemming from three ancestral populations.

Discussion

Historical biogeographic reconstructions reveal that complex kelp originated in the northeast Pacific and diversified over time by colonizing new habitats (Starko *et al.*, 2019). The kelp in the Northwest Atlantic were colonized post-glacially via oceanographic flow through the Arctic (Nielsen *et al.*, 2016; Neiva *et al.*, 2018). In the Atlantic, the kelp migrated south as far as their summer thermal maximum temperature tolerance allowed (Egan and Yarish, 1988). Since the 1980's, this region has experienced significant sea surface warming resulting in northward shifts of isotherms with serious implications for canopy forming seaweeds in both intertidal and subtidal habitats (Wilson *et al.*, 2019). In the Northwest Atlantic, our findings support a major genetic break for *S. latissima* formed by the biogeographic barrier at Cape Cod. Populations north and south of this Cape share some genetic ancestry but have also diverged over time. Despite this deep regional population structure, the genetic variation found within locations accounted for the greatest proportion of the total, indicating abundant local standing diversity available for population adaptation or breeding. Future studies will place this genomic data into a biogeographically larger context. This fundamental genetic data is useful not only for gaining a better understanding of the population structure of *S. latissima*, but also for guiding sustainable seaweed aquaculture. These results can guide management and conservation of kelp ecotypes, specifically by placing efforts to conserve certain genetics and phenotypes.

This study is part of ongoing efforts to selectively breed sugar kelp for large scale food and bioenergy production with increasing focus on germplasm banking to support future cultivation and restoration research (Wade *et al.*, 2020). Hatchery cultivation of microscopic stages of *S. latissima* are on-going and aim to improve the efficiency for selective breeding cultivars that are thermally tolerant and high yielding. Future studies of *S. latissima*, will help identify ecotypes that are best adapted for farming in the off-shore, low nutrient, environment.

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Seaweeds are a significant component of current marine aquaculture production and will play an increasing role in global food security as the human population increases rapidly over the next 30 years. Seaweed farming is analogous to plant based agriculture except that the crop is cultured in a marine environment. It differs from agriculture in that seaweeds do not require tillable land, fertilization or freshwater, which are resources that may ultimately constrain the expansion of agriculture. Seaweeds are converted into a variety of goods, such as food and nutritional supplements for humans and livestock, fertilizer, unique biochemical and biofuels. Wild and cultured seaweed also offer multiple ecosystem services, such as bioremediation for coastal pollution, localized control of ocean acidification, mitigation of climate change and habitat for other marine organisms. Incorporation of seaweeds into marine aquaculture farms in the United States (U.S.) is, however, not without its challenges. Seaweed is an unconventional food which necessitates establishing product acceptability, creating a sustained market and then balancing demand with a consistent supply for long term economic profitability. Seaweed farms also need to be developed in a manner that is compatible with wild capture fisheries, marine mammal migrations and other users of the marine environment. A comprehensive understanding of the role that cultured seaweeds play in the marine ecosystem is necessary in order to determine not only the economic value of the goods produced but also the ecosystem services offered by marine farming activities. This will result in a better understanding

of how an ecosystem approach to aquaculture incorporates the role and need for both the goods and services these macroalgae will provide.

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For centuries, humans have harvested different seaweeds for food, medicinal purposes, feed, and more recently as raw materials for industrial processes. Currently, wild harvested seaweed account for less than 5% of the total worldwide supply (FAO, 2018). The majority of seaweed production is provided via aquaculture, with 99% of the production taking place in Asia including China, Korea, Japan, Indonesia, and the Philippines, worth US $11.7 billion annually (FAO, 2018). Although seaweed aquaculture is a fast expanding industry, the global demand for seaweed-based products is surpassing the supply. Such demands necessitate either domesticating new species or further expanding the productivity of the existing leading seaweeds. According to the FAO, only a few species dominate seaweed farming, including two brown kelps, *Saccharina japonica* and *Undaria pinnatifida* (Buschmann *et al.*, 2018). To meet the present market requirements and to contribute in reducing the over-exploitation of wild stocks, experimental trials worldwide have assessed the performance of newly farmed seaweeds as potential products, particularly kelp species that have been traditionally harvested from wild populations.

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Anthropogenic disturbances, including coastal habitat modification and climate change are threatening the stability of kelp beds, one of the most diverse and productive marine ecosystems. To test the effect of temperature and irradiance on the microscopic gametophyte and juvenile sporophyte stages of the rare kelp, *Saccharina angustissima*, from Casco Bay, Maine, USA, we carried out two sets of experiments using a temperature gradient table. The first set of experiments combined temperatures between 7–18°C with irradiance at 20, 40, and 80 μmol photons m^{-2} s^{-1}. The second set combined temperatures of 3–13°C with irradiance of 10, 100, and 200 μmol photons m^{-2} s^{-1}. Over two separate 4-week trials, in 2014 and again in 2015, we monitored gametogenesis, the early growth stages of the gametophytes, and early sporophyte development of this kelp. Gametophytes grew best at temperatures of 8–13°C at the lowest irradiance of 10-μmol photons m^{-2} s^{-1}. Light had a significant effect on both male and female gametophyte growth only at the higher temperatures. Temperatures of 8–15°C and irradiance levels of 10–100 μmol photons m^{-2} s^{-1} were conditions for the highest sporophyte growth. Sporophyte and male gametophyte growth was reduced at both temperature extremes—the hottest and coldest temperatures tested. *S. angustissima* is a unique kelp species known only from a very narrow geographic region along the coast of Maine, USA. The coupling of global warming with high light intensity effects might pose stress on the early life-history stages of this kelp, although, as an intertidal species, it could also be better adapted to temperature and light extremes than its subtidal counterpart, *Saccharina latissima*.

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Cell selection technique for establishment of low salinity tolerance strain in *Pyropia tenuipedalis*†

Mahiko ABE*1, Tomomi OHASHIRA*1, Noboru MURASE*1 and Masanobu KISHIOKA*2

Abstract: *Pyropia tenuipedalis* is characterized by reddish thalli and direct budding from the shell substratum. The distribution of this species is limited to coastal areas in the Seto Inland Sea, Ise Bay and Tokyo Bay in Japan. In Yamaguchi prefecture, this species has been locally used for direct human consumption. Moreover, alanine and glutamic acid contents of *P. tenuipedalis* are three times and two times higher, respectively than those of *P. yezoensis* "nori". Yamaguchi Prefectural Fisheries Research Center began development of the mariculture technique of *P. tenuipedalis* in 2002, and succeeded in its commercialization in 2007. However, production has continued to decrease since 2012. More recently, intense disappearance of thalli is observed during January to February. One of the reasons for the production decrease is thought to be low salinity due to recent heavy rain fall supposedly associated with global climate change. The aim of this study was to develop low salinity tolerance strains of *P. tenuipedalis* through a cell selection technique. Punched out disks of thallus were cultured in a 10% seawater medium diluted with distilled water with modified 1/2SWM-III medium for 1 to 4 months. A few cells survived in this medium and were subsequently cultured in a 100% seawater medium with 1/2SWM-III. Surviving cells divided and regrew to thallus, and stock culture strains were established with self-fertilization. A field culture trial of the established low salinity tolerance strain and the conventional strain was carried out in Koto River estuary from December 2018 to February 2019. The low salinity tolerance strain showed better growth than the conventional strain, suggesting the efficacy of the cell selection technique for breeding. However, some of the thalli growing on the culture plates were suddenly shortened or disappeared in early January. Time-lapse observation with an underwater camera revealed that the disappearance of the thalli was due to predation by blackhead seabream, *Acanthopagrus schlegelii*. Immediate establishment of not only strains resistant to changing environments, but also effective measures for countering predation are necessary to increase production of *P. tenuipedalis*.

Key words: *Pyropia tenuipedalis*, breeding, cell selection, low salinity tolerance

Intoduction

Recently, fisheries production of *Pyropia yezoensis*, commonly referred to as "nori" in Japanese language, has decreased due to climate change impacts, such as seawater temperature rise and oligotrophication. Many researchers are developing or seeking new strains that have tolerances to the above-

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† *Pyropia tenuipedalis* was replaced to *Neopyropia tenuipedalis* by Yang *et al.* (2020).
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mentioned environmental stresses (Shimada, 2010; Sakaguchi, 2011). In Yamaguchi Prefecture, *Pyropia tenuipedalis*, which is closely related to *P. yezoensis*, is an indigenous species, and is a resource used to promote local industries (**Fig. 1**). As *P. tenuipedalis* has reddish thalli unlike blackish *P. yezoensis* and directly buds from the shell substratum, this species is generally called "Aka-nori" in Yamaguchi Prefecture, and the Japanese vernacular name is "Kaigara-ama-nori". Aka and Kaigara means red and shell, respectively in Japanese. The distribution of this species is limited to coastal areas in the Seto Inland Sea, Ise Bay, and Tokyo Bay in Japan, and this species is designated as an endangered species. In Yamaguchi Prefecture, this species has been used as an important local food resource for human consumption (Abe *et al.*, 2015).

The life cycle of *P. tenuipedalis* is quite different from that of *P. yezoensis* (**Fig. 2**). Matured thalli of *P. yezoensis* releases many carpospores. Carpospores germinate and grow into filamentous conchocelis. Conchosporangia are formed on conchocelis, and many conchospores are released. Conchospores divide and grow into young thalli. Young thalli of approximately 2 mm in blade length release many monospores. Monospores grow into young thalli. After release of monospores, the thalli resumes growth (Yoshida, 1993). Therefore, *P. yezoensis* can produce a large number of spores and thalli. However, matured thalli of *P. tenuipedalis* release

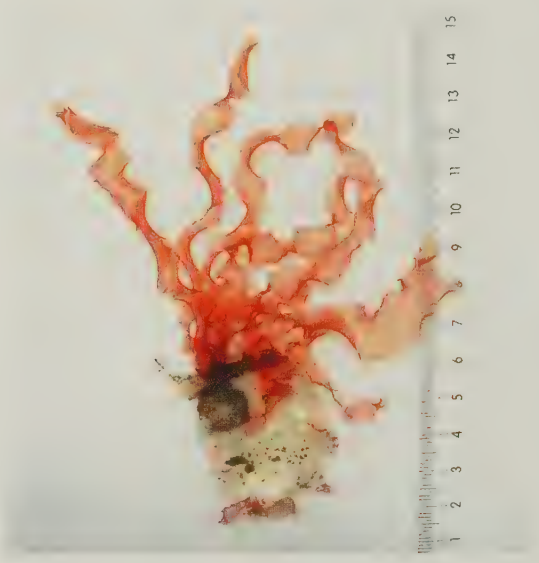

Fig. 1. Photo of *Pyropia tenuipedalis* collected in January 2011.

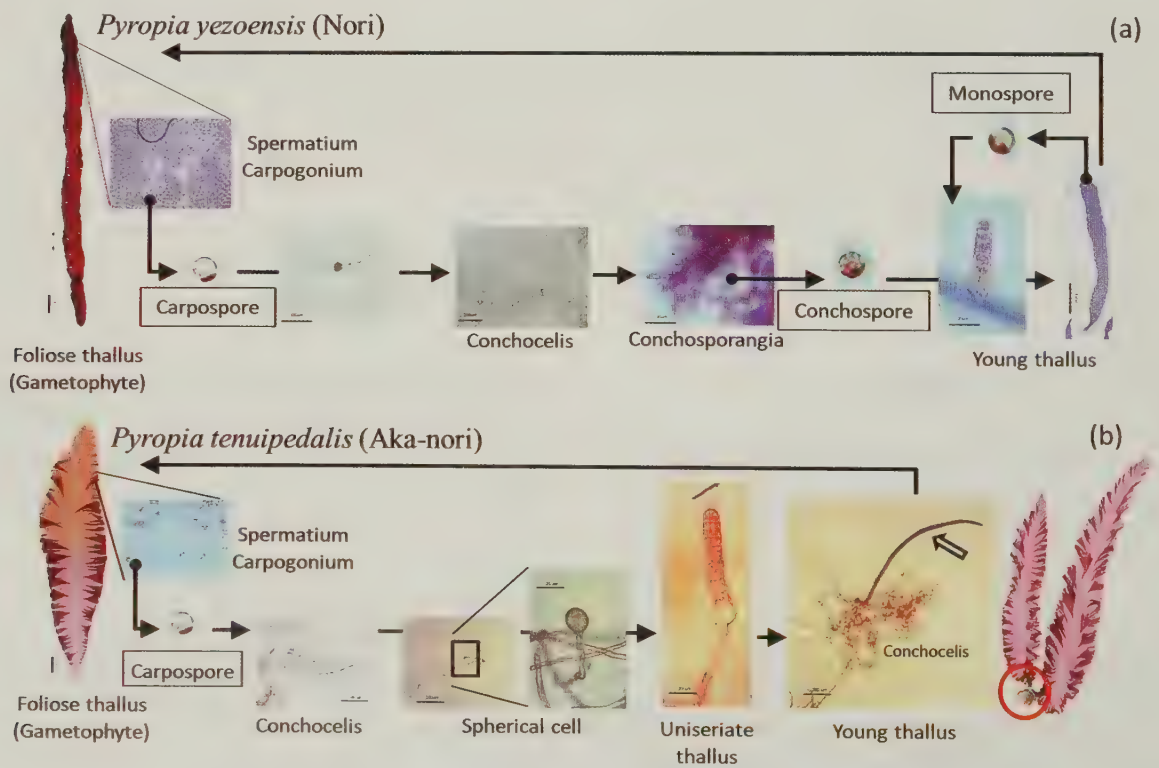

Fig. 2. Life cycles of *Pyropia yezoensis* (a) and *Py. tenuipedalis* (b).

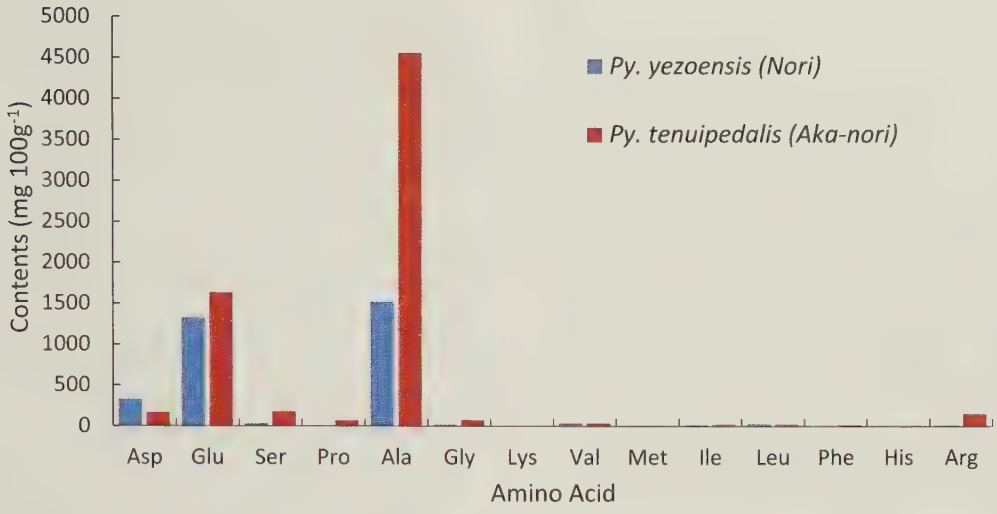

Fig. 3. Amino acid content of *Pyropia yezoensis* and *Py. tenuipedalis*.

many carpospores and becomes conchocelis, as in the case of *P. yezoensis*. However, *P. tenuipedalis* forms a spherical cell on the tip of conchocelis instead of conchosporangia. This spherical cell divides and grows into uniseriate thalli and young thalli. *P. tenuipedalis* does not have conchospore and monospore (Notoya and Kikuchi, 1993). Consequently, one spherical cell grows into one thallus.

Amino acid contents are completely different between *P. yezoensis* and *P. tenuipedalis* (**Fig. 3**). Alanine and glutamic acid contents of *P. tenuipedalis* are higher than those of *P. yezoensis*. This suggests that *P. tenuipedalis* has stronger "umami" and "sweetness" compared with *P. yezoensis*. Yamaguchi Prefectural Fisheries Research Center focused on *P. tenuipedalis* as a new mariculture species in 2002. They developed the culture plate technique for *P. tenuipedalis* mariculture and succeeded in its commercialization in 2007. This culture plate is made from calcium carbonate, and conchocelis are cultured on this plate. These culture plates are set directly on the sea bottom in the field in November every year. Thalli growing on the culture plates are harvested by hand and dried by machine from January to March, and then it become the final product. The price for the product is 1,200 Japanese yen / 10 g in dry weight. Among seaweed products in Japan, this is the most expensive.

Mariculture using the culture plates was previously carried out only in Yamaguchi Bay

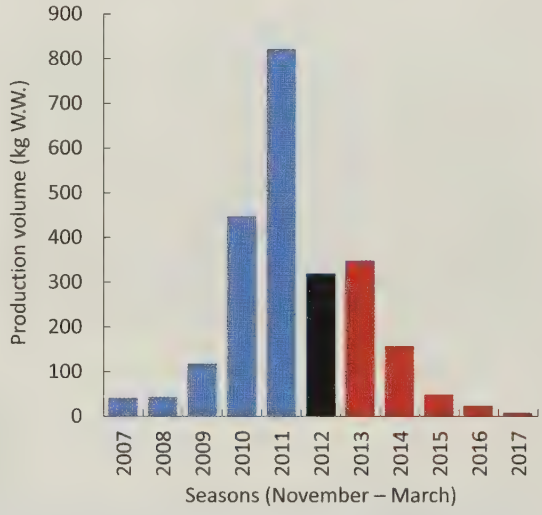

Fig. 4. Production volume of *Pyropia tenuipedalis* mariculture from November to March during 2007–2017. 2007–2011: Yamaguchi Bay (blue bar), 2012: Yamaguchi Bay and Koto River estuary (black bar), 2013–2017: Koto River estuary (red bar).

from 2007 to 2011. In 2012, it was conducted in Yamaguchi Bay and Koto River estuary. Since 2013, it has been conducted only in Koto River estuary. *P. tenuipedalis* production had the highest value in 2011 with the increase in the number of the culture plates (**Fig. 4**). However, production has continued to decrease since 2012 although the number of culture plates was kept constant. More recently, intense disappearance of thalli is observed from January to February. One of the main reasons for the production decrease is thought to be low salinity due to recent heavy rain fall. Water discharge

from the Koto River Dam during the period from December to January—the growing season for *P. tenuipedalis*—clearly increased from 2008 to 2016 by about 30% on average and 2 to 3 times in maximum instantaneous flow (Water and Disaster Management Bureau of the Ministry of Land, Infrastructure, Transport and Tourism: Database of Dams: http://mudam.nilim.go.jp/home). Therefore, we identified that low salinity tolerant strains were needed to cope with this problem.

Establishment of Low Salinity Tolerant Strain of *P. tenuipedalis*

In this study, we used a cell selection technique for establishment of a low salinity tolerant strain of *P. tenuipedalis* (Abe *et al.*, 2019). Punched out disks of thalli were cultured in the modified 1/2SWM-III medium prepared with 10% seawater (approximately 3 psu) diluted with distilled water for one to four months (**Fig. 5**). Under the low salinity conditions, although the majority of normal cells were bleached and eventually died, a few cells survived, which were then cultured in the modified 1/2SWM-III medium with 100% seawater. The surviving cells were allowed to divide and regrow to thalli, and stock culture strains were established with self-fertilization of a large size of single thalli.

Field Culture Trial of Low Salinity Tolerant Strain

A field culture trial of the established low salinity tolerant strain and the conventional strain of *P. tenuipedalis* was carried out in Koto River estuary from December 2018 to February 2019. Conchocelis of both the conventional strain and low salinity tolerant strain attached to the culture plates were set in Koto River estuary at the end of November in 2018. In addition, time-lapse observation at 1-hour intervals of culture plates was carried out with an underwater camera to determine the cause of the intense disappearance of the thalli.

The length of thalli in both strains were measured every 2 weeks from December 2018 to February 2019 (**Fig. 6**). The thallus lengths of the conventional and the low salinity tolerant strain were 1.0±1.0 mm and 1.3±1.4 mm, respectively on December 10th 2018. On December 25th, the low salinity tolerant strain grew larger (13.4±9.5 mm) than the conventional strain (7.0±5.4 mm). However, the

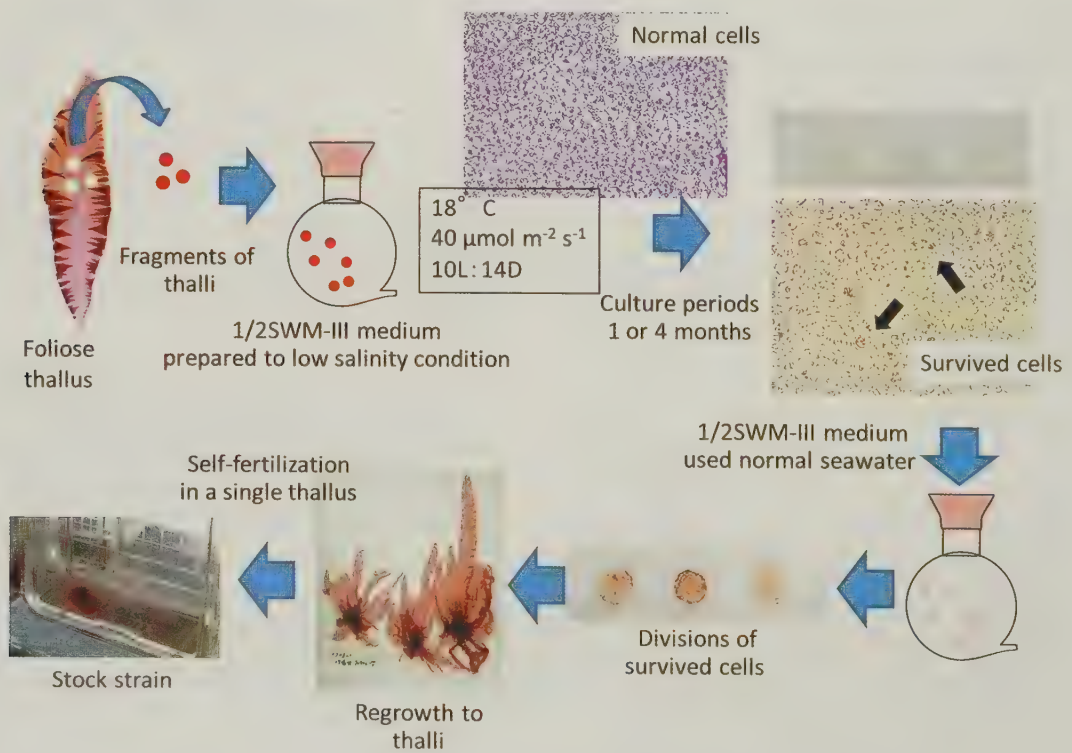

Fig. 5. Procedures of the cell selection technique used in this study.

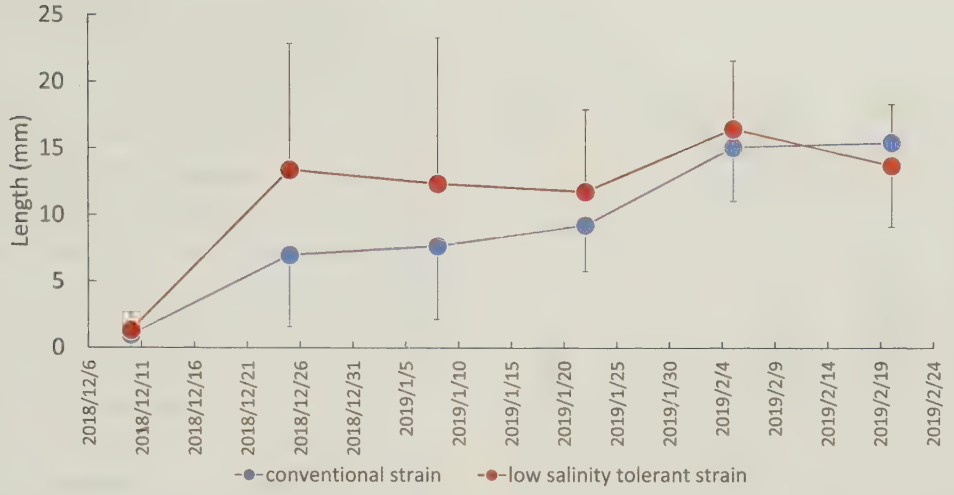

Fig. 6. The growth of thalli in the conventional and low salinity tolerant strains during the field culture trial.

length of the low salinity tolerant strain stopped growing and ranged from 12.4 ± 11.0 mm to 16.6 ± 5.1 mm from January to February. The conventional strain grew gradually through the field culture trial up to 15.5 ± 6.4 mm. Thus, the low salinity tolerance strain showed better growth than the conventional strain, suggesting the efficacy of the cell selection technique for breeding.

Intense Disappearance of the Thalli

Some of the thalli growing on the culture plates suddenly shortened and disappeared in early January. Time-lapse observation with an underwater camera revealed that the disappearance of the thalli was due to predation by blackhead seabream, *Acanthopagrus schlegelii* (**Fig. 7**). Blackhead seabream appeared in 15% of the photos taken in daytime. It was estimated that the population of blackhead seabream was large in this area from the relationship between the interval of time-lapse photography and the appearance of blackhead seabream in the photos. The intense disappearance from January 4th to 5th was remarkable (**Fig. 8**). Thalli on the culture plates were considerably shortened over one night.

We unintentionally found a countermeasure for fish predation in a field trial that was conducted to study work efficiency of the seaweed deployment. Usually, the culture plates are placed directly on the sea bottom. Therefore, activities for mariculture

Fig. 7. Photo of blackhead seabream (*Acanthopagrus schlegelii*) grazing *Pyropia tenuipedalis* growing on culture plates.

Fig. 8. Photos of *Pyropia tenuipedalis* thalli on a culture plate on January 4th and January 5th 2019.

Fig. 9. Specimens of *Pyropia tenuipedalis* collected on January 7th 2019, including: (a) thalli on the culture plates set directly on the sea bottom, and (b) thalli on the culture plates placed within a crab basket. Bars indicate 5cm.

management, such as cleaning sediments on the culture plates and harvesting thalli, are restricted during low tide at the spring tide due to limited accessibility. In order to enable the operator to perform the activities regardless of the tidal height, the culture plates were placed within a crab basket that can be lifted up on the boat. As part of the study, we compared the growth between thalli set directly on the sea bottom and those placed within a crab basket (**Fig. 9**). We found that the thalli within a crab basket grew better and that the crab basket was effective in physically protecting the thalli from fish predation.

Conclusion

In Yamaguchi Prefecture, *P. tenuipedalis* has been used as a local fisheries resource, and mariculture of this species was intended as a unique local industry. However, intense disappearance of the thalli has been observed from January to February for the past few years, which is a hindrance to the successful industrialization. One of the reasons for the production decrease was thought to be low salinity due to recent heavy rainfall. We established low salinity tolerant strains by developing the cell selection technique for breeding. A field culture trial showed the established low salinity tolerant strain had better growth than the conventional strain in the early culture period.

Intense disappearance of *P. tenuipedalis* due to predation by omnivorous fish were observed by time-lapse observation with underwater camera set in the field culture trial site. For *P. tenuipedalis* mariculture using culture plates, it is advisable to physically protect the seaweed from fish predation in a cage.

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Annotated Bibliography of Key Works

(1) Abe M., Murase N., Hatama T., Shikano Y., and Kanai T., 2015: New record of *Pyropia tenuipedalis* from Koto river estuary, Yamaguchi prefecture. *J. Nat. Fish. Univ.*, **63**, 244–248. (in Japanese with English abstract)

We collected reddish foliose thalli from Koto River estuary, Yamaguchi Prefecture. In order to identify the species, we carried out field collections of the foliose thalli, morphological observation in culture and PCR-RFLP analysis using the two regions of the partial mitochondrial DNA. As for the morphological survey, the spherical cells were formed at the tips of conchocelis and developed to foliose thalli. The fragment patterns of this species in the PCR-RFLP analysis matched with *Pyropia tenuipedalis*. In the present study, the foliose thalli collected at Koto River were identified as *P. tenuipedalis*, which is an endangered species distributed along the coast of Japan. This species was newly recorded from Koto River estuary, Yamaguchi Prefecture.

(2) Nakayama T., Abe M., Murase N., and Shikano, Y., 2017: Influence of salinity on growth of red alga *Pyropia tenuipedalis* and *Pyropia yezoensis* foliose thallus. *Aquacult. Sci.*, **65**, 321–330. (in Japanese with English abstract)

Pyropia tenuipedalis is a new culture species in Yamaguchi Prefecture, Japan. The habitat of this species is more brackish as compared with that of *P. yezoensis*, which is the common species in Japanese *nori* mariculture. In this paper, the authors investigated the relationship between salinity and growth of *P. tenuipedalis* and *P. yezoensis*. It was revealed that *P. tenuipedalis* tolerates lower salinity in comparison with *P. yezoensis*.

(3) Abe M., Murase N., Hatama T., Shikano Y., and Kanai T., 2017: Environmental characteristics of *Pyropia tenuipedalis* (Miura) Kikuchi et Miyata growing at Yamaguchi Bay, Yamaguchi Prefecture. *J. Nat. Fish. Univ.*, **65**, 19–29. (in Japanese with English abstract)

This study surveyed environmental characteristics of *Pyropia tenuipedalis* foliose thallus growing in Yamaguchi Bay, Yamaguchi Prefecture from November to March, 2010–2014. Young foliose thallus of this species appeared in December and grew to their maximum length from January to March. In March, mature thalli were observed. Water temperature from November to March was usually within the range of 6–16°C, but the temperature was lower in early December in 2012 than other years. Light reaching the growing depth of this species was 6.2±3.1% of the level at water surface at high tide. The concentration of dissolved inorganic nitrogen at the sampling site was within the range of 8.8–68.3 μmol L^{-1}, which was approximately 10 times higher than the level that is assumed to cause the discoloration of *P. yezoensis*. Water temperature reduced at a rate of 0.33°C day^{-1} from November to December in 2012–2013, which was faster than the rate in other years. In this study, it was suggested that the growth of thalli from spherical cells was inhibited by a long- or short-term rapid water temperature decrease that occurred from November to December.

(4) Abe M., Murase N., Nakae M., Nakayama T., Nakagawa M., and Shikano Y., 2018: Water temperature characteristics in growth of filamentous thallus and formations of spherical cell, uniseriate and foliose thallus of *Pyropia tenuipedalis* (Miura) Kikuchi et Miyata. *J. Nat. Fish. Univ.*, **66**, 81–88. (in Japanese with English abstract)

We investigated the water temperature effects on growth of filamentous thallus and formations of spherical cell, uniseriate and foliose thallus of *Pyropia tenuipedalis* with culture experiments. Optimal growth of filamentous thalli was observed at 20°C. Moreover, optimal water temperatures for formations of spherical cells, uniseriate and foliose thalli were 20°C, 15–20°C and 15°C, respectively. Optimal water temperatures of each life stage were different. At 10°C, the formations of uniseriate and foliose thalli were suppressed. At 25°C, almost all thalli had morphological abnormalities. Furthermore, decreasing temperature to 15°C from 20°C enhanced formation of uniseriate and foliose thalli. It was thought that low production in 2012 resulted from suppressed formation of uniseriate and foliose thalli at less than 10°C.

(5) Murase N., Abe M., Fukudome K., Nakagawa M., and Shikano Y., 2018: Influence of temperature on the growth of red alga *Pyropia tenuipedalis* thalli. *J. Nat. Fish. Univ.*, **66**, 215–220. (in Japanese with English abstract)

This study was designed to clarify the optimal temperatures for growth in uniseriate thalli and foliose thalli of *Pyropia tenuipedalis* under laboratory culture at 5°C intervals from 10°C to 25°C or 30 °C. The optimal temperature for uniseriate thalli developed from a spherical cell at the two-cell stage were 15°C and 20°C. The optimal temperature for foliose thalli allowed to develop from young blades with a length of approximately 6 cm was 15 °C. It was suggested that the shift in the optimal temperatures from uniseriate to foliose thalli stages was related to the seasonal reduction of water temperature from autumn to winter.

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New diets with potential for enhancement of juvenile bivalve seed production and culture techniques

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Abstract: Environmental changes are now having a negative impact on a number of aquatic organisms. In the decades after the mid-1980s, annual catch of bivalves such as the Manila clam (*Ruditapes philippinarum*) continues to decrease drastically in the coastal waters of Japan, and several causative factors have been reported. To conserve the bivalve resources, the promotion of efficiency and stabilization of seed and juvenile bivalve production is needed in addition to the promotion of marine ecosystem and habitat recovery. Under these circumstances, a persistent problem is insufficient food supply for juvenile bivalves because of the difficulty in stable production of diet microalgae at low cost and a deficiency of microalgae that possesses all the necessary dietary requirements. Thus, there is a compelling need for the development of dietary-supplements and/or new species of diet microalgae that contain the essential nutritional properties, and of alternative feeds to replace live microalgal diets. In this mini-review, new diet microalgae, a dietary-supplement, and original feeds using microencapsulation, enzymatic decomposition and fermentation technology with potential for the enhancement of juvenile bivalve seeding production and culture techniques are introduced.

Key words: alginate hydrolysate, *Eutreptiella eupharyngea*, lipid-walled microcapsule, marine silage, seeding production of bivalves

Introduction

Human activities including greenhouse gas emissions have led to increasing global temperatures, perturbed regional weather patterns, rising sea levels, acidifying oceans, changed nutrient loads, and altered ocean circulation (Brierley *et al.*, 2009). Such a changing environment is now having a negative impact on a number of aquatic organisms (Brierley *et al.*, 2009; Rodolfo-Metalpa *et al.*, 2011; Kroeker *et al.*, 2013). In particular, there are growing concerns about impacts of environmental change on microalgae, which are the essential primary producers in marine and freshwater aquatic ecosystems. Boyce *et al.* (2010) analyzed the data on available ocean transparency measurements and *in situ* chlorophyll observations to estimate the time dependence of phytoplankton biomass at local, regional and global scales since 1899, and concluded that global phytoplankton concentration has declined over the past century. They also indicated that these fluctuations are strongly correlated with basin-scale climate indices, whereas long-term declining trends are related to increasing sea surface temperatures (Boyce *et al.*, 2010).

In Japan, several studies have indicated dramatic decreases in fisheries production and environmental and ecosystem changes in coastal areas such as long-term decreasing trends of phytoplankton biomass and nutrient concentration, long-term changes in species composition of phytoplankton, and increasing sea surface temperatures (Wanishi, 2005; Noda and Yukihira, 2013; Abe, 2017; Yamamoto, 2019; Nishiwaka, 2019; Kaeriyama *et al.*, 2019). In the decades after the mid-1980s, annual catch of bivalves, especially the Manila clam (*Ruditapes philippinarum*),

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in coastal waters of Japan continues to decrease drastically. Though the precise cause is unclear, a number of factors affecting the dramatic decrease of the clam species (including overfishing) have been suggested (Hamaguchi *et al.*, 2002; Paillard, 2004; Park *et al.*, 2006; Tsutsumi, 2006; Matsukawa *et al.*, 2008; Toba *et al.*, 2013; Toba, 2017). Furthermore, a wide variety of studies have aimed at conserving the clam resource (Dang *et al.*, 2010; Paul-Pont *et al.*, 2010; Shigeta and Usuki, 2012; Suzuki *et al.*, 2012; Usuki *et al.*, 2012; Kobayashi *et al.*, 2012; Ikushima *et al.*, 2012; Sakurai *et al.*, 2012; Hasegawa *et al.*, 2012; Nakagawa *et al.*, 2012; Sakami and Higano, 2012; Houki *et al.*, 2015; Hanyu *et al.*, 2017; Hata *et al.*, 2017; Houki *et al.*, 2018). To conserve the clam resource, however, promotion of efficiency and stabilization of seed production and juvenile clam culture are needed.

In this mini-review, new diet microalgae, a dietary-supplement, and original feeds using microencapsulation, enzymatic decomposition, and fermentation technology with potential for the enhancement of juvenile bivalve seeding production and culture techniques are reviewed.

Dietary Effect of a New Diet Microalgae and Mixed Algal Diets on Juvenile Bivalves

Microalgae are used as live feeds for larval or juvenile bivalves, crustaceans and other invertebrates in addition to the rotifer *Brachionus plicatilis*, which is fed to larval fish. Thus, diet microalgae should contain essential nutritive constituents, be nontoxic, be appropriately sized for ingestion, and possess digestible cell walls to allow the nutrients to be absorbed after ingestion (Becker, 2004). In addition, it is important that the microalgae can be produced in large quantities at low cost. So far, more than 40 species of microalgae have been isolated and analyzed to produce better aquaculture feeds, and these species are undergoing cultivation as pure strains in intensive systems (Becker, 2004). However, there is no microalgae that possesses all the necessary requirements of an ideal diet alga. Therefore, the development of such new diet microalgae could be crucially important for the enhancement of seed production and culture of juvenile bivalves.

As described in the Introduction, a variety of studies have sought to conserve wild *R. philippinarum* populations while providing a stable market supply through developing clam culture. A persistent problem, however, is insufficient food supply for juvenile clams exceeding a shell length of 1 mm (especially in late fall and spring) since clams have high food requirements, and low temperature slows the growth of the diet microalgae. Therefore, the development of new species of diet microalgae that can grow well outdoors at low water temperatures and that possess the essential nutritive constituents will greatly benefit juvenile clam culture. Recently, Yamasaki *et al.* (2019) isolated the marine euglenophyte *Eutreptiella eupharyngea* from a pond used for extensive phytoplankton cultivation at the Yamaguchi Prefectural Fisheries Research Center (Yamaguchi, Japan) in January 2013. The study reported from the results of both laboratory and outdoor experiments that *E. eupharyngea* could grow well at water temperatures ranging from 4 to 25℃ , but could not grow at 30℃ . Furthermore, Yamasaki *et al.* (2019) demonstrated that the dietary effect of *E. eupharyngea* per dry weight, on juvenile *R. philippinarum* of more than 1.5 mm shell length, exceeded that of the diatom *Chaetoceros neogracile*— a known suitable diet alga for juvenile clams. These findings were attributed to the high nutritional value of *E. eupharyngea* as typified by its high protein and sugar content and high content ratio of n-3 fatty acids such as eicosapentaenoic and docosahexaenoic acid and n-6 fatty acids such as arachidonic acid. In addition, *E. eupharyngea* (**Fig. 1**) appears to have a stronger dietary effect on bigger clams (e.g., more than 1.5 mm in shell length) since *E. eupharyngea* cells (cell length: 35–70 μm; cell width: 7.5–12 μm, Walne *et al.*, 1986) are bigger than other diet microalgae such as *C. neogracile* (cell size: <10 μm). Thus, *E. eupharyngea* shows considerable potential to become a new diet alga for the seed production and culture of juvenile bivalves in late fall and spring.

A multitude of studies suggest that mixed microalgal diets may provide a better balance of essential nutrients to bivalves. Rivero-Rodríguez *et al.* (2007) analyzed the relative fatty acid

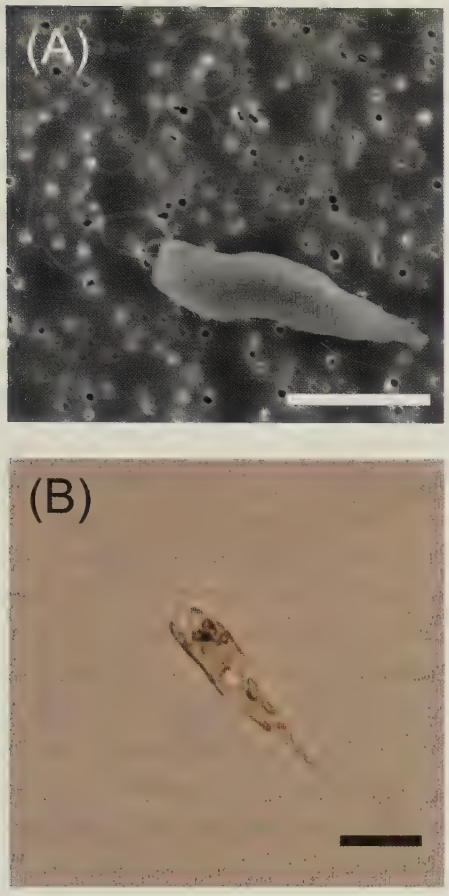

Fig. 1. A scanning electron microscope (SEM) photograph (A) and a micrograph (B) of *Eutreptiella eupharyngea*. The scale bars indicate 10 μm.

composition of the diatoms *Chaetoceros calcitrans*, *C. muelleri*, and *Phaeodactylum tricornutum*, as well as the haptophyte *Isochrysis galbana* and the prasinophyte *Tetraselmis suecica*, and reported that these microalgae contained a high proportion of either EPA (C20 : 5) or DHA (C22 : 6). Furthermore, they examined the dietary contribution of these five algal species when provided as mono- or bi-algal diets to juveniles of the mangrove oyster (*Crassostrea corteziensis*), and concluded that *C. calcitrans* provided the best diet, probably due to its high AA (C20 : 4) content (Rivero-Rodríguez *et al.*, 2007). In addition, Ronquillo *et al.* (2012) examined the effect of mixed microalgal diets on the growth and fatty acid profile of European flat oyster (*Ostrea edulis*) juveniles. The authors indicated that dietary effect of the mixture of the eustigmatophyte *Nannochloropsis oculata* and the haptophyte *Pavlova lutheri*, which have had higher levels of polyunsaturated fatty acids (PUFAs), was higher than that of other combinations of diet microalgae. However, Geng *et al.* (2016) examined the effects of four different microalgae, *C. calcitrans*, *I. galbana*, *N. oculata* and *Diacronema viridis*, on the growth of juvenile ark shells (*Tegillarca granosa* Linnaeus), and demonstrated that the best feeding effects were observed with the mixture of all four microalgae, and binary algal diets were second best. Recently, Liu *et al.* (2016) evaluated the nutritional value of eight species of microalgae for larvae and early post-set juveniles of the Pacific geoduck Clam (*Panopea generosa*), and reported that a balanced mixture of various dietary nutrients was important. In particular, they suggested that the ratios between n-3 and n-6 fatty acids, and between EPA and DHA, are especially crucial (Liu *et al.*, 2016). Polyunsaturated fatty acids (PUFAs), especially the n-3 fatty acids eicosapentaenoic acid (EPA, C20 : 5n-3) and docosahexaenoic acid (DHA, C22 : 6n-3) play an important role in bivalve growth and development (Volkman amd Brown, 2005; Martínez-Fernández *et al.*, 2006). Thus, the fatty acid composition of diet microalgae might be one of the most important factors for bivalve growth and development.

Nevertheless, content and/or composition of these fatty acids could be affected by microalgal growth rates, environmental conditions, and growth phases (Richmond, 1986). The main environmental factors affecting the growth and the nutritive constituents of diet microalgae are light levels, nutrients, temperature, pH, and salinity (Chu *et al.*, 1996; Tzovenis *et al.*, 1997; Zhu *et al.*, 1997). The impact of increasing water temperature and ocean acidification should also be considered.

Enhancement of Dietary Effect on Juvenile Bivalves by the Dietary Supplements

Jórgensen (1983) observed that clams take up dissolved organic matter (DOM) in seawater through epidermal tissue in the mantle and gills. Welborn and Manahan (1990) showed that larvae of the bivalve *Crassostrea gigas* (Pacific oyster) can take up dissolved glucose, maltose, cellobiose, and cellotriose, but not rhamnose or maltotriose. In addition,

Uchida *et al.* (2010) reported that the growth rate of soft tissue in *R. philippinarum* was significantly promoted by supplementing a diet of the diatom *C. calcitrans* with glucose at concentrations of 10 and 100 mg L^{-1}. Furthermore, Taga *et al.* (2013) reported the high dietary effects of the raphidophyte *Heterosigma akashiwo* (known as a harmful algal species) on juvenile *R. philippinarum* and suggested that the acidic sugars found in microalgal cells may be one of the important factors determining the growth of juvenile clams. Thus, we focused on alginate—a known acidic sugar.

Alginate is a natural acidic linear polysaccharide that is composed of α-L-guluronate and β-D-mannuronate (uronic acids) residues. This carbohydrate occurs in the cell walls of brown algae, such as *Saccharina japonica* and *Undaria pinnatifida*. Alginate and its hydrolysates are currently used in a wide range of commercial products because of their safety, low price, and bioactivities. Since alginate is poorly soluble in water, we focused on the dietary effect of alginate hydrolysates which are water soluble and thus usable by clams. Results showed that shell-length growth of juvenile *R. philippinarum* was significantly promoted by supplementing a diet of *C. neogracile* with alginate-hydrolysates (AHs) of at least 1 mg L^{-1}. The most effective concentrations of AHs were 2 to 4 mg L^{-1}, but shell length growth in groups given AHs only without *C. neogracile* was significantly inhibited (Yamasaki *et al.*, 2015). In addition, Yamasaki *et al.* (2016) demonstrated that growth of adult clams (initial average shell length [±SD], 15.7±0.3 mm) was dramatically promoted by supplementing a diet of the diatom *C. neogracile* with AHs at 4 mg L^{-1}, and metabolomics indicated that each of the states of starvation, food satiation, and sexual maturation of *R. philippinarum* has a characteristic pattern in the metabolite profile (Yamasaki *et al.*, 2016).

A few kinds of *Chaetoceros* spp. are widely used for clam culture, but the cost of cultivating *Chaetoceros* spp. is expensive. *Nannochloropsis* spp. are used for cultivation of a various marine molluscs, crustaceans, and zooplankton and can be produced in large quantities at low cost (Zhang *et al.*, 2001). However, the dietary effect of *Nannochloropsis* spp. on juvenile bivalves is not always sufficient as compared to other diet microalgae. Accordingly, we tried to enhance the dietary effect of *Nannochloropsis* sp. on the shell length growth of juvenile *R. philippinarum* by AHs supplementation. Yamasaki *et al.* (2018) reported that shell length and total weight of clams were significantly promoted by supplementing a diet of *Nannochloropsis* sp. at the concentration of 30×10^4 cells mL^{-1} with AHs at the concentration of 4 mg L^{-1} as compared with the groups given *Nannochloropsis* sp. Thus, a combination of *Nannochloropsis* sp. and AHs will be useful to shorten the rearing period of clams at low cost since shell length growth of the clams fed *Nannochloropsis* spp. added with AHs were faster than that of clams fed more costly *Chaetoceros* spp.

Particulate organic matter (POM) appears to contribute to improvements in the quality of bivalves. To improve the quality of the freshwater Clam (*Corbicula japonica*) by rearing in a short period, Nojiri *et al.* (2018) examined the effect of various carbohydrates on the increment of glycogen content, and of the hyperosmotic stress on the amino-acid uptake. As a result, Nojiri *et al.* (2018) showed that rice powder was effective for increasing glycogen content at the concentration of 0.1g/L, and suggested that insoluble carbohydrate was suitable for the increment of glycogen content in the freshwater clam. Furthermore, they observed that glycine was most effectively absorbed in the freshwater clam, followed by proline, alanine and glutamic acid under hyperosmotic stress for 24 h, and concluded that the quality of *C. japonica* could be improved in a short period by feeding rice powder and rearing in palatable amino acids under osmotic stress.

New Approaches in the Development of Original Feeds for Bivalves

There is a compelling need for the development of alternative feeds to replace live microalgal diet because of the difficulty in stable production of diet microalgae at low cost and a deficiency of microalgae that possesses all the necessary requirements for a diet alga. In this section, several new approaches in the development of original feeds using microencapsulation, enzymatic decomposition and

fermentation technology for bivalves are introduced.

Recently, several studies have suggested that microcapsules, which can easily be produced in large quantities, have highly customizable physical characteristics and contents, and are stable for long term storage (Aldridge *et al.*, 2006; Costa *et al.*, 2011). Thus, microcapsules have promise as alternative feeds to replace a live microalgal diet. Willer and Aldridge (2017) demonstrated that a new form of microencapsulated diet known as BioBullets (BioBullets Ltd., Cambridge, UK) can successfully be ingested by the blue mussel (*Mytilus edulis*). Furthermore, Willer and Aldridge (2019) demonstrated that the use of microencapsulated feed, which were lipid-walled microcapsules containing 50% powdered *Schizochytrium* algae by weight and manufactured by BioBullets (BioBullets Ltd.), can lead to major improvements in survivorship and growth in juvenile European flat oysters (*Ostrea edulis*).

Several studies have also suggested the viability of marine silage (MS) and single cell detritus (SCD; Uchida and Murata, 2002; Pérez Camacho *et al.*, 2004; Uchida *et al.*, 2004). MS and SCD can easily be produced in large quantities, have a suitable size for ingestion by filter feeder such as bivalves, and are stable for long term storage without remarkable loss of particulate products. Thus, MS and SCD have promise as alternative feeds to replace live microalgal diet. Pérez Camacho *et al.* (2004) showed that SCD prepared from *L. saccharina* using enzymatic and bacterial decomposing activities has some dietary effect on the clam *Ruditapes decussatus*. Though shell length growth of the clam spats fed *C. gracilis* was higher than those fed PS, Kalla *et al.* (2008) reported that spheroplasts prepared from *Porphyra yezoensis* (Rhodophyta) using enzymatic decomposition had some dietary effect on the *R. philippinarum* spat. In addition, Uchida and Murata (2002) suggested "marine silage" prepared from *U. pinnatifida* as a novel fisheries-diet, which is produced from the combination of conversion of seaweed to SCD and induction of lactic acid fermentation utilizing activities of a lactic acid bacterium and yeast. Furthermore, Uchida *et al.* (2004) demonstrated that MS prepared from *U. pinnatifida* had a limited but positive dietary effect on Japanese pearl oyster (*Pinctada fucata martensii*) spat.

Conclusion

Research and development of new diets such as diet microalgae, dietary supplements, and original feeds using microencapsulation, enzymatic decomposition and fermentation technology are rapidly developing. In the near future, efficiency and stabilization of seed production and juvenile clam culture may be achieved by a combination of new diets and live algal diets. Therefore, these techniques may have important implications for clam culture, and could contribute to the conservation of the wild clam resources and a stable market supply. Further studies are needed to develop a process for practical utilization of these techniques for seed production and juvenile clam culture.

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Annotated Bibliography of Key Works

(1) Yamasaki Y., Taga S., and Kishioka M., 2015: Preliminary observation of growth-promoting effects of alginate hydrolysates on juvenile Manila clams, *Ruditapes philippinarum*. *Aquaculture Res.*, **46**, 1013–1017.

Several studies have suggested that certain types of sugars are potentially a good supplement for growth of bivalves such as *Ruditapes philippinarum*. We observed the dietary effects of a harmful raphidophyte *Heterosigma akashiwo* on juvenile clams and suggested that the acidic sugars in the phytoplankton might be an important factor determining the shell length growth of clams because total sugar and acidic sugar content of *H. akashiwo* were higher than other diet microalgae. Therefore, we focused on alginate known as one of the acidic polysaccharides, and showed that shell-length growth of juvenile clams (average shell length: 432 to 507 μm) was significantly promoted by supplementing the diatom *Chaetoceros neogracile* (40,000 to 80,000 cells mL^{-1}) with alginate-hydrolysates (AHs) of at least the concentration of 1 mg/L. In addition, the most effective concentrations of AHs were 2 to 4 mg L^{-1}.

(2) Yamasaki Y., Taga S., Kishioka M., and Kawano S., 2016: A metabolic profile in *Ruditapes philippinarum* associated with growth-promoting effects of alginate hydrolysates. *Sci. Rep.*, **6**, 29923.

We demonstrated that shell length growth of *Ruditapes philippinarum* (average shell length: 15.7 mm) was significantly promoted by supplementing the diatom *Chaetoceros neogracile* (80,000 cells mL^{-1}) with alginate-hydrolysates (AHs) at the concentration of 4 mg L^{-1}. Furthermore, metabolomics indicated that clams in the groups given *C. neogracile* with AHs at the concentration of 4 mg L^{-1} actively utilized excess carbohydrate for the development of reproductive tissue. On the other hand, clams in the groups given *C. neogracile* only were actively growing through the use of their adequate carbohydrate resources. Thus, supplementation of AHs with the algal diet may be an effective way to shorten the rearing period of clams.

(3) Yamasaki Y., Ishii K., Taga S., and Kishioka M., 2018: Enhancement of dietary effect of *Nannochloropsis* sp. on juvenile *Ruditapes philippinarum* clams by alginate hydrolysates. *Aquaculture Rep.* **9**, 31–36.

The eustigmatophyte *Nannochloropsis* sp. is widely used in the aquaculture industry because this species can be produced on a large scale at low cost. However, *Nannochloropsis* sp. has less dietary effect on juvenile bivalves compared with other diet algae such as the diatom *Chaetoceros neogracile* and the haptophyte *Diacronema* (=*Pavlova*) *lutheri*. In this study, the use of alginate-hydrolysates (AHs) to enhance the dietary effect of *Nannochloropsis* sp. on juvenile *Ruditapes philippinarum* (average shell length: 1,090 μm) was attempted. As a result, enhancement of the dietary effect on shell-length growth of juvenile clams was observed in the groups given *Nannochloropsis* sp. (300,000 cells mL^{-1}) with AHs at the concentration of 4 mg L^{-1}. Hence, the enhanced dietary effect of a combination of *Nannochloropsis* sp. and AHs will be useful to shorten the rearing period of *R. philippinarum*.

(4) Yamasaki Y., Ishii K., Hikihara R., Ishimaru M., Sato F., Taga S., Kishioka M., Matsunaga S., Shikata T., Abe M., Kato S., Tanaka R., and Murase N., 2019: Usefulness of the euglenophyte *Eutreptiella eupharyngea* as a new diet alga for clam culture. *Algal Res.*, **40**, 101493.

Microalgae are an essential feed source for seed production of bivalves such as *Ruditapes philippinarum*. However, there is a deficiency of microalgae that can provide a stable supply of nutrient-rich feed at low water temperatures during winter and spring. To develop a new diet of microalga that can grow well outdoors at low water temperatures and possesses the essential nutritive constituents, we focused on the euglenophyte *Eutreptiella eupharyngea*, which was isolated from a pond used for extensive phytoplankton cultivation at the Yamaguchi Prefectural Fisheries Research Center (Yamaguchi, Japan) in January 2013. As a result, this species grew well at water temperatures of 10–25℃, but could not grow at 30℃. Furthermore, the dietary effect of *E. eupharyngea* per dry weight on juvenile *R. philippinarum* (average shell length: 1,426 μm) exceeded that of the diatom *Chaetoceros neogracile*. These findings are attributable to the high nutritional value of *E. eupharyngea* as typified by its high protein and sugar content and high content ratio of n-3 fatty acids such as eicosapentaenoic and docosahexaenoic acid and n-6 fatty acids such as arachidonic acid.

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Development of a sustainable diet for Japanese white trevally *Pseudocaranx dentex* juveniles

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Extended Abstract

The Japanese white trevally *Pseudocaranx dentex, Shimaaji* in Japanese, (Bloch & Schneider, 1801) is a highly prized carangid fish species for sushi and sashimi that is distributed in tropical and temperate waters around the world, except in the eastern Pacific region. There is an increased demand for the white trevally due in part to the increase in artificially hatched fingerlings which are only cultured in Japan. Overall, published studies on the nutritional requirements of juvenile white trevally are limited and there is ongoing research being conducted to determine the most suitable protein sources for developing a practical diet.

In Japan and around the world, the primary commodity utilized for protein in aquaculture feed formulations is fish meal (FM), which is produced from the mass-catch of pelagic fish species. FM tends to have high cost oscillations, and its high level of inclusion in marine fish diets tends to have negative consequences on the environment, including phosphorus pollution from the effluent of fish fed FM-based diets. Soybean meal (SBM) is an alternative protein source that is a promising candidate for FM replacement in juvenile white trevally diets. In general, marine fish species tend to exhibit varied sensitivity to SBM due to the presence of phytic acid and other antinutritional factors, which can contribute to issues related to low palatability, further leading to poor growth due to deficiency of indispensable amino acids such as methionine and lysine. However, the amino acid, palatability and anti-nutritional factors associated with SBM could be overcome with the addition of supplemental crystalline amino acids, taurine, phytase supplementation, and palatability enhancers by developing an appropriate feed formulation the white trevally.

In the aquaculture industry, the flavor attractant krill meal (KM) is utilized as a high-level protein source and palatability agent for juvenile marine fish. In order to formulate a low-cost and environmentally sustainable diet for juvenile white trevally, it is necessary to find a suitable replacement for KM that serves as a high-quality protein source, has a well-balanced amino acid profile, and works well as a flavor attractant. Furthermore, it is necessary to develop a feed formulation that completely replaces FM in practical diets. Land animal by-products have potential as alternative feed ingredients in formulated diets for marine fish. One of the most noteworthy animal by-products is poultry by-product meal (PBM), which consists of rendered waste material generated from poultry slaughterhouses and processing plants. PBM, which has a good amino acid balance and high protein content has yet to be tested in in practical SBM-based diets for juvenile white trevally as a protein source and palatability agent.

Three feeding trials conducted at the Aquaculture Research Institute of Kindai University from 2018—2020 demonstrated that high levels of SBM replacement by FM in combination with palatability enhancers KM or PBM, indispensable amino acids Lys and Met, taurine, and phytase in the diets provided high utility when fed to juvenile Japanese white trevally. While SBM has been mostly considered as an inferior protein source to FM when included in marine fish aquaculture

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diets, the results of the three feeding trials revealed great potential for SBM as an FM replacer. It is evident that Japanese white trevally is an excellent candidate for alternative protein sources derived from plants including SBM. The diets tested provided high survival and adequate growth rates to warrant further development of practical diets for this species. Fish fed diets containing higher levels of SBM had a lower environmental impact than fish fed with FM-based diets, which highlights the ecological benefits of utilizing alternative protein sources. It is recommended that future research focuses on determining a mechanism to understand why juvenile white trevally achieve better growth performance with higher levels of SBM than FM in the diet. The results from the three feeding trials mentioned above will be described in future publication.

Annotated Bibliography of Key Works

(1) Biswas A., Araki H., Sakata T., Nakamori T., Kato K., and Takii K., 2017: Fish meal replacement by soy protein from soymilk in the diets of red sea bream (*Pagrus major*). *Aquac. Nutr.*, **23**, 1379–1389.

The results of this study introduce the ecological benefits of replacing FM by soy-based products in juvenile red sea bream, such as reduced phosphorus discharge to the environment. This feeding experiment was conducted at the Aquaculture Research Institute of Kindai University, Japan using the same methods that were utilized in the research presented in our extended abstract.

(2) Jirsa D., Davis A., Stuart K., and Drawbridge M., 2011: Development of a practical soy-based diet for California yellowtail, *Seriola lalandi*. *Aquac. Nutr.*, **17**, e869-e874.

This study was carried out in an effort to develop an environmentally sustainable and cost-effective diet for California yellowtail juveniles using soy products to replace fish meal. This research used a similar approach in terms of feed formulation, specifically investigating the utility of soybean meal and soy protein concentrate. Interestingly, fish fed diets containing soybean meal outperformed fish fed soy protein concentrate, which further influenced our choice of testing the utility of conventional soybean meal on white trevally juveniles. This research was conducted in the United States.

(3) Kader Md. A., Bulbul M., Koshio S., Ishikawa M., Yokoyama S., Nguyen B.T., and Komilus C. F., 2012: Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, *Pagrus major*. *Aquaculture*, **350–353**, 109–116.

The authors found that in red sea bream juveniles, nutrient utilization, body composition and blood parameters were improved or not significantly affected by replacing fish meal with soybean meal in combination with flavor attractant supplementation. Growth performance results from this experiment express a similar trend to the results presented in our abstract, in which soybean meal diets outperformed the fish meal control diets in white trevally. This research was conducted in Japan.

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